Oxygen exchange in galls of *Eurosta solidaginis* (Diptera: Tephritidae)

by

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Abstract

Hypoxia in plant tissue should affect animals living within. Gallmakers stimulate their plant hosts to produce the gall they inhabit and feed on, and also influence the gall phenotype for other adaptations, such as defense against predators. The potential for hypoxia in galls of *Eurosta solidaginis* was studied in the context of potential adaptations to gall oxygen level, using a combination of direct measurement, mathematical modelling, and respirometry on both gallmakers and hosts. Modelling results suggested mild hypoxia tolerable to the larva persists for most of the growth season, whereas more severe hypoxia may occur earlier in fully-grown young galls. Field data from one of the two years studied showed hypoxia more severe than expected, and coincided with adverse weather conditions and high larval mortality. The hypoxia may be related to host response to adverse weather. Whether hypoxia directly caused larval mortality requires further study.

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Chapter 1: Introduction

The diversity of hypoxic insect habitats

Obtaining oxygen for respiration is critical for all aerobic organisms, including insects. As one of the most diverse animal classes, insects inhabit a great variety of habitats, many of which have lower oxygen levels (i.e. are hypoxic) compared to atmospheric air at sea level. Some environments are hypoxic due to simple physics: oxygen diffuses through water slowly compared to in air, and even oxygen-saturated water may contain 20-30 times less oxygen than air under similar temperature and pressure, due to differences in the solubility or carrying capacity of these different media (Hoback & Stanley, 2001). Therefore, temporary submergence in water would be considered hypoxic for terrestrial insects. Aquatic insects often have adaptations which increase the efficiency of oxygen extraction from water or allow them to breathe air directly. Oxygen content also decreases as altitude increases due to low atmospheric pressure. Nevertheless, insects are still found at over 4000-5000 m above sea level (Sømme *et al.,* 1996; Williams *et al.,* 2010).

Hypoxic conditions also occur in small spaces where air flow is restricted, especially when oxygen is consumed by the substance surrounding the space in addition to the inhabitant itself. Besides ground burrows (Anderson and Ultsch 1987), habitats of this type also include the medium on which insects are feeding, which may be metabolically active as tissue of a living organism or due to microbial activity. Examples include dung, carrion, storage areas for agricultural products such as grain or fruit, inside host tissue (for parasites), and decaying logs (Hoback and Stanley, 2001). Gas exchange

in small spaces may be further restricted by water, which may take the form of seasonal flooding (Hoback *et al.* 1998), tidal flooding (Wyatt, 1986) or melting snow and ice in the spring (Paim and Beckel, 1964).

Insect adaptations to hypoxia

The need to exploit resources in semi-permanently hypoxic habitats, or at least to survive and escape temporary hypoxia, has led to diverse adaptations exhibited by insects. In general, insects recover better from exposure to severe hypoxia and anoxia than vertebrates (Harrison *et al.,* 2006). They often reduce their activity level and therefore metabolic rates, and may employ anaerobic pathways for basic energy requirements (e.g. Zerm *et al.*, 2004). However, reducing activity may be unsuitable for insects that exploit resources while under prolonged hypoxia, because doing so would interfere with feeding and growth. Reducing activity may also be unfeasible for insects that seek to physically escape from hypoxia due to limited mobility. So, in addition to a means to reduce energy requirement, adaptations are also dedicated to enhancing oxygen delivery required for energy production in hypoxia.

One way of obtaining extra oxygen in an otherwise hypoxic medium is by securing access to air for its high oxygen content. Some aquatic insects, such as certain members of the Hemiptera and some dipteran larvae, have breathing tubes that extend above the surface to take in air directly (e.g. Abdel-Malek, 1949; Blinn *et al.,* 1982). Botfly larvae that inhabit feeding medium also use this strategy (Lane *et al.,* 1987). Air trapped underwater as bubbles may be important for flooding survival (Zerm *et al.,* 2004), and it is possible to prevent flooding by maintaining surface tension at a small burrow opening

or by directly blocking the burrow (Wyatt, 1986). In many aquatic and semi-aquatic species, air trapped by specialized water-repellent body hairs forms a bubble around their body (plastron) that facilitates gas exchange with surrounding water. Some species are even able to maintain submergence indefinitely using the plastron (Flynn and Bush, 2008). At the very least, animals may move away from detrimental hypoxia if different degrees of hypoxia exist in the feeding medium (Hoback and Stanley, 2001). Indeed, exposure to hypoxia may elicit exploratory behavior, resulting in *Drosophila melanogaster* larvae moving away from food (Wingrove & O'Farrell, 1999).

In chronic hypoxia, capacity of gas exchange can also be augmented by developmental plasticity. This mainly involves increasing the branching and/or diameter in the tracheal system (Loudon, 1989), which could be triggered by products resulting from activation of HIF (Hypoxia Inducible Factor) pathways (Harrison *et al.*, 2006). However, these changes may be unable to compensate for the effects of hypoxia completely, leading to delayed development and reduced survival (e.g. Greenberg and Ar, 1996; Houlihan, 1974).

Despite the physiological challenges of hypoxia, some insects have also adapted to live for extended periods, at least during certain life stages, in severe hypoxia. A well-known example is the chironomid larva which often inhabits bottom layers of water bodies where prolonged anoxia is possible. Even species like *Chironomus riparius*, considered to have poor hypoxia tolerance, maintains a constant metabolic rate until oxygen concentration decreases to 0.8 ± 0.1 kPa (Penttinen and Holopainen, 1995). A more resilient species, *Chironomus plumosus*, showed 50% mortality after 205 days in anoxia (Nagell and Landahl, 1978). Besides typical strategies that involve increasing

ventilation or reducing metabolism, the Chironomidae also have haemoglobin in their haemolymph that binds to oxygen very effectively under hypoxia. Human and sheep haemoglobins have P_{50} (oxygen partial pressure at which haemoglobin is 50% saturated with oxygen) of 26 torr (1 torr ≈ 0.133 kPa), and high-altitude camelids show P_{50} values between 17-22 torr (Storz, 2007). Meanwhile, P_{50} of chironomid haemoglobin may be below 1 torr (Burmester and Hankeln, 2007). Certainly, aquatic insects may adapt to hypoxia better than terrestrial insects in the first place, due to the prevalence of hypoxia in aquatic habitats, but high tolerance to hypoxia is also observed in terrestrial species. *Orthosoma brunneum* larvae, which normally inhabit decaying beech logs, can survive 1.5% oxygen at room temperature (unspecified in study) while moving and feeding normally. However, they were unable to survive below 0.6% oxygen for over 10 days (Paim and Beckel, 1964).

Question: would galls be hypoxic?

Galls are abnormal growths of plant tissue that develop due to stimuli from parasites such as insects, mites, fungi and bacteria. Gall tissue acts both as food and protection. However, having to live inside the gall for extended periods, gallmakers must also withstand whatever conditions they encounter inside the gall. Due to elevated metabolism during growth or insufficient oxygen supply in the environment, plant tissue may experience hypoxia (Geigenberger, 2003). Extra tissue growth in galls leads to a higher volume of tissue, which increases both oxygen consumption and distance for gas transport. In addition, plant metabolic rate may also increase during growth. Hence, the possibility of hypoxia developing in gall tissue should be higher than in healthy gall-bearing plant tissue, and gallmakers may face hypoxia inside their own galls.

Being confined inside the gall, some adaptations for coping with hypoxia may be unavailable to gallmakers. Specifically, if the gall imposes hypoxia, the gallmaker is unlikely to escape the adverse condition without leaving the gall. Doing so compromises the gall's protection, which may be even more detrimental than withstanding hypoxia depending on the intensity of predation or other sources of mortality. On the other hand, galls form due to the host responding to stimuli produced by the gallmaker which may be a result of the gallmaker's genotype. Galls therefore fall under the extended phenotype concept (Abrahamson and Weis, 1997). If genetic variation in the stimulus leads to variation in gall phenotype that influences gallmaker fitness, then the stimulus is subject to natural selection. So, if the ancestral gall imposes a hypoxic condition on the gallmaker, is there potential for novel adaptations against gall hypoxia to evolve via modification of the gall-inducing stimulus, leading to changes in the gall phenotype? In order to continue this line of research, the hypothesis that galls constitute a hypoxic microhabitat needs to be tested first.

Raman (2007) suggested that hypoxia at insect feeding sites could play a role in gall development. Ramírez (1997) studied breathing adaptations of fig wasps in genera *Apocryptophagus*, *Apocrypta* and *Sycophaga*. These wasps develop inside modified flowers described as galls in the fig syconia, and males travel between flowers in search for females. Young syconia are filled with liquid, which likely poses greater challenge to respiration than the plant tissue does. *Apocryptophagus* and *Sycophaga* males have filament-like peritremes on abdominal spiracles which emerge last when the males leave the current flower, likely maximizing time taken in gas exchange. *Apocrypta* males carry gas bubbles on broad, concave peritremes. This example illustrates potential respiratory

challenges faced by insects living in a plant organ. However, the fig flower gall tissue itself was considered a less hypoxic environment than the liquid-filled syconia, so this study cannot support the potential of hypoxia in galls with no obvious liquid space. Subsequently, Haiden *et al.* (2012) studied oxygen levels in galls under the context of how photosynthesis influences the gallmaker. Their subject was *Trichilogaster acaciaelongifoliae*, a gallmaker used as a biocontrol agent against its host *Acacia longifolia.* Oxygen level in gall tissue was found to be below 5%, and the larval chamber showed more severe hypoxia of 2.4%. Influence of photosynthesis was minimal and restricted to near the gall's surface.

The research organism

The goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera: Tephritidae), parasitizes two goldenrod species, *Solidago altissima* (or *S. canadensis var. scabra*) and *S. gigantea.* Uhler (1951) documented its life history in detail, and Abrahamson and Weis (1997) provided a summary of ecological and evolutionary perspectives regarding its interaction with host and predators. Eggs are inserted into goldenrod leaf buds via a sharp ovipositor (Figure 1-1E) in late spring. The larva burrows into the apical meristem, and releases chemicals that induce the growth of distinctive spherical galls up to 3 cm in diameter (Figure 1-1A). Like most gall-inducing arthropods, *E. solidaginis* produces a prosoplasmic gall that differentiates into distinctive tissue zones (Figure 1-2), instead of the calluslike kataplasmic galls (Abrahamson and Weis, 1997). The gall is divided into four tissue layers from the inside out (Weis *et al.,* 1989):

1. The **nutritive pith** fed upon by the larva. Cells resemble normal pith cells but are smaller.

2. A thin-walled **parenchyma** layer rich in resin canals and vascular strands arranged radially.

3. Parenchyma with a thick cell wall, referred to as the **cortex** layer for distinction from the thin-walled parenchyma. This is the thickest layer and is responsible for the majority of variation in gall size. Cells in the layer contain little cytoplasm.

4. The vascular column surrounding the stem, referred to as the **skin** layer to avoid confusion with the vascular strands. This layer is not significantly modified from the normal stem tissue.

The larva occupies a chamber inside the nutritive pith, which expands during larval growth due to tissue consumption. The larva passes through three instars in total, and excavates an exit tunnel up to the skin in late fall (Figure 1-1C). Pupation occurs in spring, and the adult breaks through the remaining gall skin by inflating the ptilinum, a fluid-filled sac on the head (Figure 1-1D). Adults do not feed, are poor fliers, and only live for a couple of weeks for mating and oviposition.

There have been three main areas of research on *E. solidaginis*: the evolution of populations that specialize on different host species (host races) as the beginning of speciation; the ecological interaction between the gallmaker and its host and predators; and how the larva survives freezing conditions during winter. Some findings from

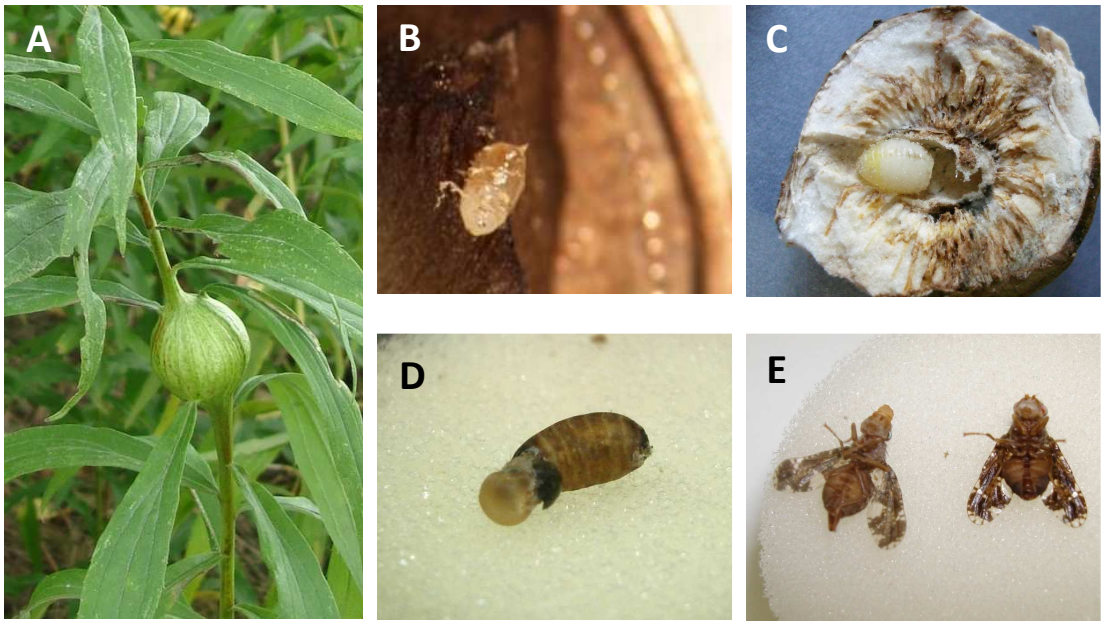

Figure 1-1: Major stages in the *Eurosta solidaginis* life cycle. A: Gall on a *Solidago* stem. B: Young larva extracted from its gall, placed on a Canadian one-cent coin (diameter 19.05 mm) as scale reference. C: Mature larva in a dead gall. The exit tunnel has been excavated. D: Adult emerging from pupa, with ptilinum still inflated. E: Ventral view of adult female (left) and male (right), distinguishable by the female's ovipositor at the end of the abdomen. All photos were taken during this study.

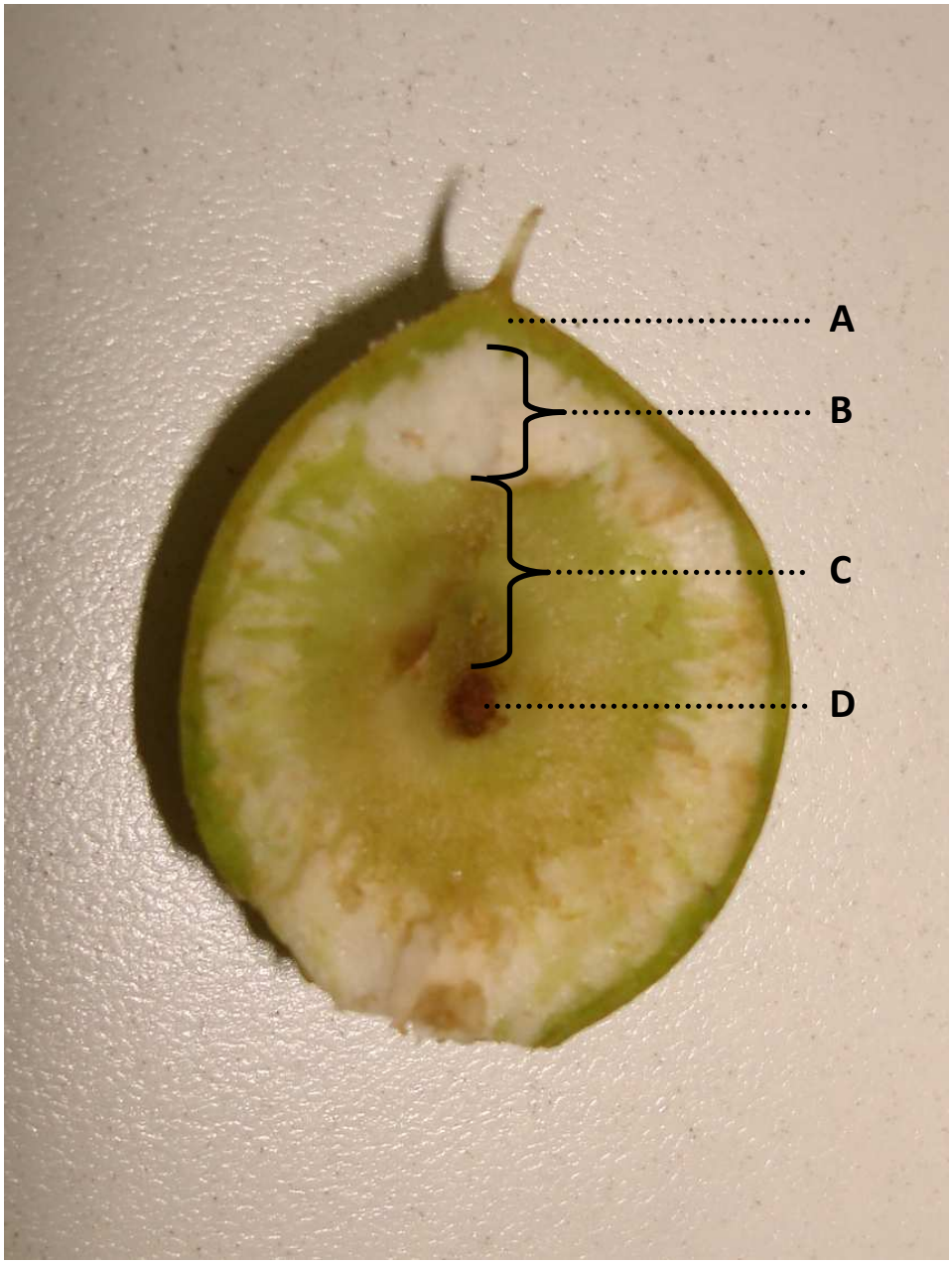

Figure 1-2: Vertical cross section of a gall. A: Vascular layer (skin). B: portion of cortex layer where transition to the dry, spongelike texture has started. C: Cytoplasm-rich tissue, including young cortex, thin-walled parenchyma, and the nutritive layer lining the larva's chamber (D). Gall examined in late July, 2010.

previous studies are especially relevant to the study of potential gall hypoxia and adaptation against it. First of all, selection on gallmaker genotype via heritable gall phenotype is possible in *E. solidaginis*. Gall size is affected by insect genotype (Weis and Abrahamson 1986), and is also subject to selection from predators. The parasitic wasp *Eurytoma gigantea* oviposits on *E. solidaginis* larva by penetrating the gall wall. Since ovipositor length limits the size of galls the adult wasp can penetrate, smaller galls are more vulnerable to wasp penetration (Weis *et al.*, 1985). On the other hand, downy woodpeckers (*Picoides pubescens*) and black-capped chickadees (*Poecile atricapillus*), both gall predators during winter, can easily penetrate even the largest galls. Unlike *E. gigantea*, these birds more frequently attack large galls, likely due to easier visual detection or greater likelihood of finding the more nutritious gallmaker larva instead of wasp larva (Abrahamson and Weis, 1997). Therefore, gallmaker genotype that affects gall size is under selection from predation, and the direction of selection depends on the relative strength of predation from the two predator types (Abrahamson and Weis, 1997). This also means any other selection pressure on gall size may face interference from selection due to predation.

Overwintering larva are exposed to freezing temperatures, because the gall itself has no insulating properties (Layne, 1993). *E. solidaginis* larvae is able to delay freezing by reducing the freezing point of its body fluid (supercooling) and also tolerates freezing (Irwin and Lee, 2002), and the mechanism for its freeze tolerance has been subject to extensive research. Frozen insects have to tolerate prolonged tissue hypoxia due to the lack of muscle-assisted ventilation and the slow diffusion of oxygen through the frozen tissue. Frozen *E. solidaginis* larvae showed elevated levels of hypoxia-inducible factor-1α

(HIF-1α) transcripts, and so did larvae exposed to anoxia (Morin *et al.* 2005). During winter, if oxygen delivery into the tissue is already severely limited, external hypoxia is unlikely to exert additional selection for better response to hypoxia. In addition, goldenrods overwinter as underground rhizomes whereas aboveground ramets (individual flowering shoots) die off. Even if the dead, non-respiring gall tissue still impedes oxygen diffusion, the exit tunnel excavated by the larva in late autumn might act as a shorter route for diffusion. The larva's metabolism is also greatly depressed at low temperatures, which helps to conserve energy to be used by non-feeding adults and therefore potentially enhancing fitness (Irwin and Lee, 2003). Low metabolism would reduce oxygen consumption. Therefore, winter hypoxia inside the gall would be inconsequential to the larva, if it is present at all. Hence, this study focused on summer and autumn. However, if the larva experiences hypoxia at higher temperatures, adaptations that help the larva survive freezing hypoxia may also be adaptive at higher temperatures.

Source of research organism

Galls used in this study were gathered from goldenrod patches near Brock University, St. Catharines (43°11′N 79°14′W, Figure 1-3). *Solidago gigantea,* characterized by the lack of trichomes on the stem (Abraham and Weis, 1997), was not found on sites visited during this study, including goldenrod patches off-campus. Hence all plants used were assumed to be *Solidago altissima* or at least part of the *S. canadensis* species complex. Other *Solidago* stem gallmakers occur in the area, but their spindle-shaped galls are easily distinguished from ball galls produced by *E. solidaginis*. Gall collection was spread over different goldenrod patches to avoid exterminating galls at any particular patch. Location differences were not among the goal of this study. Efforts

Figure 1-3: *Eurosta solidaginis* and host plant distribution at Brock University. Area A had been an open field habitat at least since at least year 2003. Area B covered goldenrod growths near the forest path across campus leading to the DeCew hydroelectric plants. Galls were found in both areas. Goldenrods were also found in areas C and D, but galls were not observed during the study. Aerial photo © GeoEye from Google Earth, image dated March 18, 2010.

were made to use galls from the same patch for the same experiment. However, materials collected as by-product of other experiments may be used for any experiment in order not to exhaust available galls early in any given research season.

Research objectives

This study was carried out to address the following question: Does hypoxia occur in the galls of *Eurosta solidaginis* larvae? If so, then how well does the larva tolerate the oxygen level found in its gall? The question was addressed via these experiments:

1. Estimate the oxygen level(s) below which the larva is unable to maintain a stable metabolic rate (critical P_{O_2} or P_C) and/or activity using flow-through respirometry techniques (Chapter 2).

2. Given the constraints of *in situ* sampling on wild galls, develop a mathematical model to characterize potential changes in gall oxygen level throughout the growth season (Chapter 3).

3. Directly measure oxygen levels inside living galls in the field using fluorescence-based oxygen probes, and relate the results to modelling results and other biotic or abiotic conditions (Chapter 4).

From these results, my thesis provides insights into whether hypoxia affects *E. solidaginis* larvae during their development.

Chapter 2: The larva's tolerance to hypoxia and other respiratory characteristics

Introduction

Defining hypoxia with respect to the normal oxygen level in the medium may be a simple and straightforward standard, but it is also of little use without comparison to the organism's oxygen requirement (Hoback and Stanley, 2001). Such a comparison is especially important for this study, due to the context of possible gallmaker adaptation to hypoxia (see Chapter 1). Tolerance of hypoxia is often described by critical oxygen partial pressure (critical P_{O_2} or P_C), the oxygen level below which the animal is unable to maintain a stable metabolic rate, and its oxygen consumption starts to decrease as ambient oxygen level is reduced further (Pörtner and Grieshaber, 1993). Adult and pupal insects generally have P_Cs below 5 kilopascals (kPa), which is lower than P_Cs of some small vertebrates (Greenlee and Harrison, 2004). Meanwhile, juvenile insects may show P_Cs as high as 14-18 kPa (Greenlee and Harrison, 2004). Insects that feed in hypoxic habitats could maintain a normal rate of oxygen consumption down to oxygen levels of 1-2 kPa, which enables them to function in severe hypoxia (e.g. Holter and Spangenberg, 1997; Penttinen and Holopainen, 1995). In practice, P_C is often estimated by measuring carbon dioxide (CO_2) production at ever decreasing oxygen levels (e.g. Greenlee *et al.*, 2007; Klok *et al.*, 2010). By assuming a constant respiratory quotient (RQ), carbon dioxide production can be utilized as a measure of metabolic rate in place of oxygen consumption. The test subject can be provided with CO_2-free gas by treating the gas source with chemicals that absorb CO_2 (scrubbing), so that CO_2 content in the gas output

from the subject's enclosure represents total production by the subject. This is easier than comparing the difference of oxygen content in the gas flow before and after consumption by the subject.

Temporary hypoxia in the range of hours or days is generally well-tolerated by insects (Harrison *et al.,* 2006). During temporary hypoxia, such as those encountered during daily or seasonal flooding, metabolic depression that reduces demand for oxygen is a common strategy, and energy requirement can be supplemented with anaerobic pathways (Hoback and Stanley, 2001). However, metabolic depression usually involves reduction of activity level, which may interfere with feeding and growth. This may not be a problem most of the time, but there are situations when insects need to exploit resources that are inherently hypoxic due to biological activity, such as rotten fruits, dung, rotting wood and living host tissue (Hoback and Stanley, 2001). Accumulation of resources is important for fitness by influencing traits such as fecundity (Honěk, 1993) and fighting ability (Lailvaux *et al.,* 2005). If metabolic depression is the primary adaptation against hypoxia in the feeding environment, then the advantage of being able to exploit resources in hypoxia would be compensated by slow assimilation of food. Therefore, better capacity for normal activity level under hypoxia should be adaptive for insects that develop under prolonged hypoxia through better gas delivery mechanism and/or capacity for anaerobic metabolism.

In this chapter, P_C of *Eurosta solidaginis* larvae is estimated using flow-through respirometry techniques on larvae facing progressively more severe hypoxia. The results would be used to evaluate whether hypothetical (Chapter 3) and directly measured (Chapter 4) gall oxygen levels pose a challenge for the larva, and therefore may select for

better adaptation against hypoxia. In preparation for calculations in Chapter 3, the rate of oxygen consumption by the larva was also estimated at the beginning of the progressive hypoxia trials.

Method

Equipment and material

In order to estimate P_C, CO_2 production (as a proximate estimate for energy expenditure) by healthy larvae exposed to gradually decreasing oxygen levels was measured in a flow-through respirometer, based on the procedure used by Klok *et al.* (2010) for *Drosophila melanogaster*. The equipment was assembled as shown in Figure 2-1. Gases used in the experiment were first mixed in a flask where they were pumped through 2% NaOH solution and a tube of soda lime (Alfa Aesar®, 8-12 mesh, with indicating color) to remove existing CO_2 and humidify the gas to reduce the potential for stress on the larva from dehydration. Nitrogen (99.7% purity) came from a Parker Balston N2-14 nitrogen generator, and air came from a compressed air supply. Nitrogen produced by the generator was indistinguishable from nitrogen taken from a liquid nitrogen tank (assumed to be 100% pure) using Neofox FOXY-AF oxygen probes. Oxygen content of the gas mixture was controlled by adjusting the relative flow rates of the two gas sources, using two Omega FMA-114 regulators connected to a FMA5DPV-LA/HA control module. The total flow rate of air and nitrogen into the flask remained at 160 mL/min when relative flow rates were adjusted.Both the flask and the sample chamber were placed in a modified ice chest, the temperature in which was controlled by water pumped from a water bath through a fluid-circulation radiator in the chest. A small electric fan

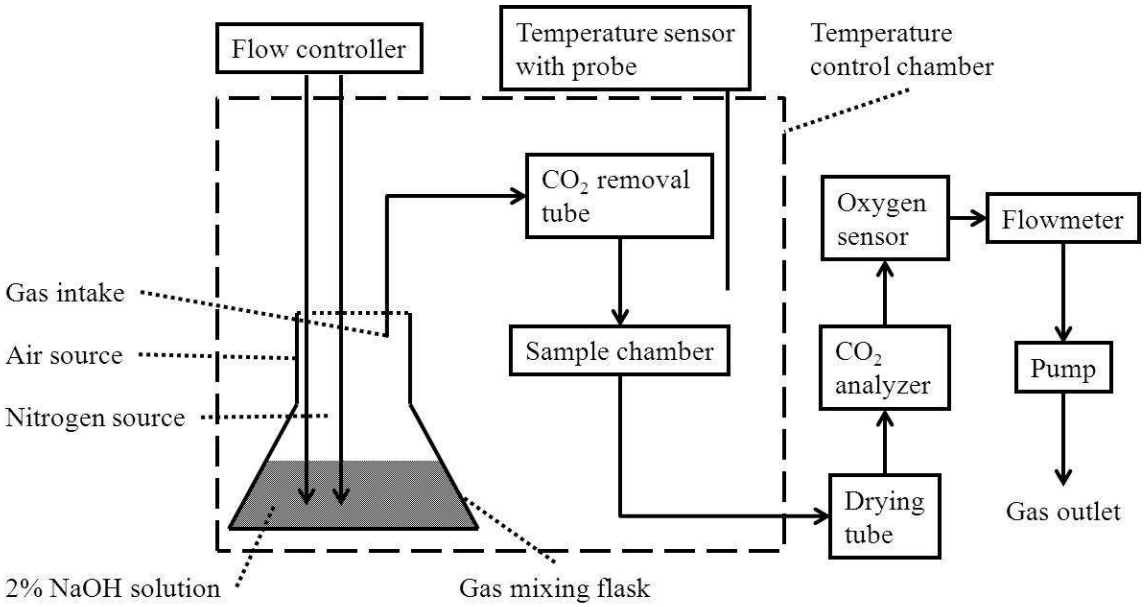

Figure 2-1: Flow-through respirometer used to estimate P_C of *E. solidaginis* larva. Solid lines with arrows indicate gas tubes and direction of air flow.

circulated air in the chest to promote temperature change. Temperature in the chest was monitored with a Sable TC-1000 thermometer with a type T probe. Gas leaving the sample chamber was dried in a tube of Drierite®, then fed into a CO_2 analyzer (Sable Systems CA-2A set to range of 1000 ppm, calibrated with 500 ppm CO_2 mixed in a Li-Cor® 6000-01 gas calibration cylinder) and an oxygen sensor (Ametek N-22M sensor connected to S-3A/I oxygen analyzer, calibrated with room air and lab nitrogen supply). The Hiblow C-5BNS-0110 air compressor that moves the gas mixture through the sample chamber and gas analyzers was connected after the analyzers, so that pressure produced by the compressor would not interfere with the analyzers. A mechanical flowmeter (calibrated with a bubble flowmeter) connected to the outlet of the compressor limited the flow rate of the gas mixture from the mixing flask through the sample and gas analyzers at 8 mL/min, which is lower than the flow rate of the two gas sources into the flask (160 mL/min). Therefore the gas mixture continuously escaped from the flask and would not be contaminated by outside air.

After placing the larva in the sample chamber, air was first passed through the respirometer for 30 minutes for habituation and to establish equilibrium. Then the oxygen concentration being mixed in the flask was reduced from the atmospheric concentration of 20.9% once every 5 minutes, in a step-wise fashion. The rate of reduction was 5% for the first 2 intervals, and then by 1% every time until the setpoint reached zero. Flow control was set back to all air flow after 30 minutes of exposure to all nitrogen flow. At the end of each trial, atmospheric gas pressure was recorded from the CO_2 analyzer's built-in sensor, in order to convert oxygen measurements from percentage in raw data to kPa. Since CO_2 was removed from the incoming gas mixture by NaOH and soda lime,

CO_2 level in output gas recorded by the sensor represents net production by the larva. Average CO_2 level during the one minute before oxygen level first fell below 20% was recorded as CO_2 production in normoxia. Larvae retrieved from the chamber were measured again for wet weight, checked for activity as a sign of survival, and then dried in an oven for dry weight measurements.

Temperature, oxygen and CO_2 sensors were connected to a BIOPAC MP150 system, from which data were collected throughout the experiment at a rate of 1.25 readings per second using AcqKnowledge 3.8.1 software package under Windows XP. The oxygen sensor used could not be calibrated to correctly display oxygen level near zero. When pure nitrogen was fed into the sensor and with the calibration dial turned to minimum, the display could read about 0.2-0.7% instead. Therefore, all oxygen readings were corrected linearly by assuming the lowest oxygen reading taken represented 0.3% oxygen (corresponding to the nitrogen generator's expected 99.7% purity) and the highest oxygen reading was accurately calibrated to read 20.95% when receiving compressed air. Oxygen level was recorded to reflect the changing composition of the gas mixture from atmospheric oxygen level (all air) to near anoxia (all generator nitrogen), so the actual magnitude may not be as important.

E. solidaginis larvae were gathered on campus. Most galls were taken from Area B (Figure 1-3), but larvae found in galls taken for field measurements (Chapter 4, Area A) or elsewhere in St. Catharines were also used in order to minimize the number of galls taken from the local population. After extraction from their gall (to be weighed in their respective experiments), non-Area-B larvae were sometimes used at a later date. In this

case, they were covered with moist tissue paper and kept at room temperature, and survival was confirmed by observing activity before they were used.

Estimation of P_C with two-part regression

P_C was estimated following a repeated regression approach documented by Yeager and Ultsch (1989). Corresponding O_2 and CO_2 data, sorted by increasing O_2 level, would be divided into two parts at an arbitrary data point, and linear regression would be calculated for each part. The residual sum of squares (RSS) of the two lines would then be calculated. This procedure would be repeated many times, using a different data point in the set as the point of division each time, except those taken at very high or very low oxygen levels and are therefore not expected to be near the P_C. The division that minimizes the sum of RSS between the two lines would be considered the best fit, and oxygen level at the intersection of the two lines would be the P_C (Figure 2-2). Calculations were performed in R 2.12.2. The script used (Appendix I) was based on the BASIC program developed by Yeager and Ultsch (1989), adapted for the R environment and data storage formats (Appendix II) used in this study. Output values of the script were given in percentage of oxygen, which were converted to the corresponding value in kPa using air pressure readings taken at the end of each trial from the CO_2 analyzer's built-in sensor. O_2-CO_2 plots were automatically generated by the script and could be visually examined for irregularities.

Analyzing larval activity

The transparent glass sample chamber was covered with a plastic lid lined with aluminium foil, which reflects light emitted by an infrared sensor under the chamber.

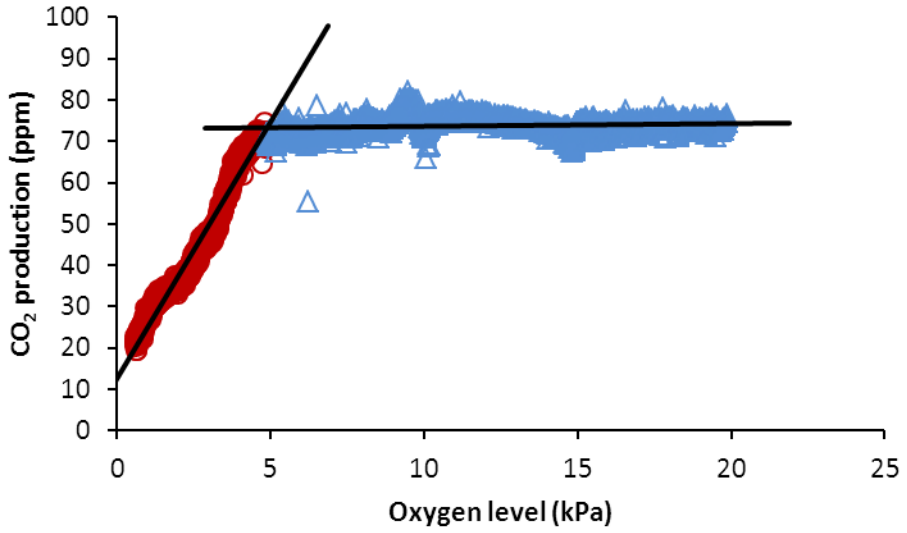

Figure 2-2: Plotted output from a typical CO_2 P_C estimation trial. The change in marker color represents the division that minimizes the residual sum of squares (RSS) between two regression lines. Intersection of the two lines represents the estimated breakpoint in the curve, indicating the beginning of metabolic depression in hypoxia.

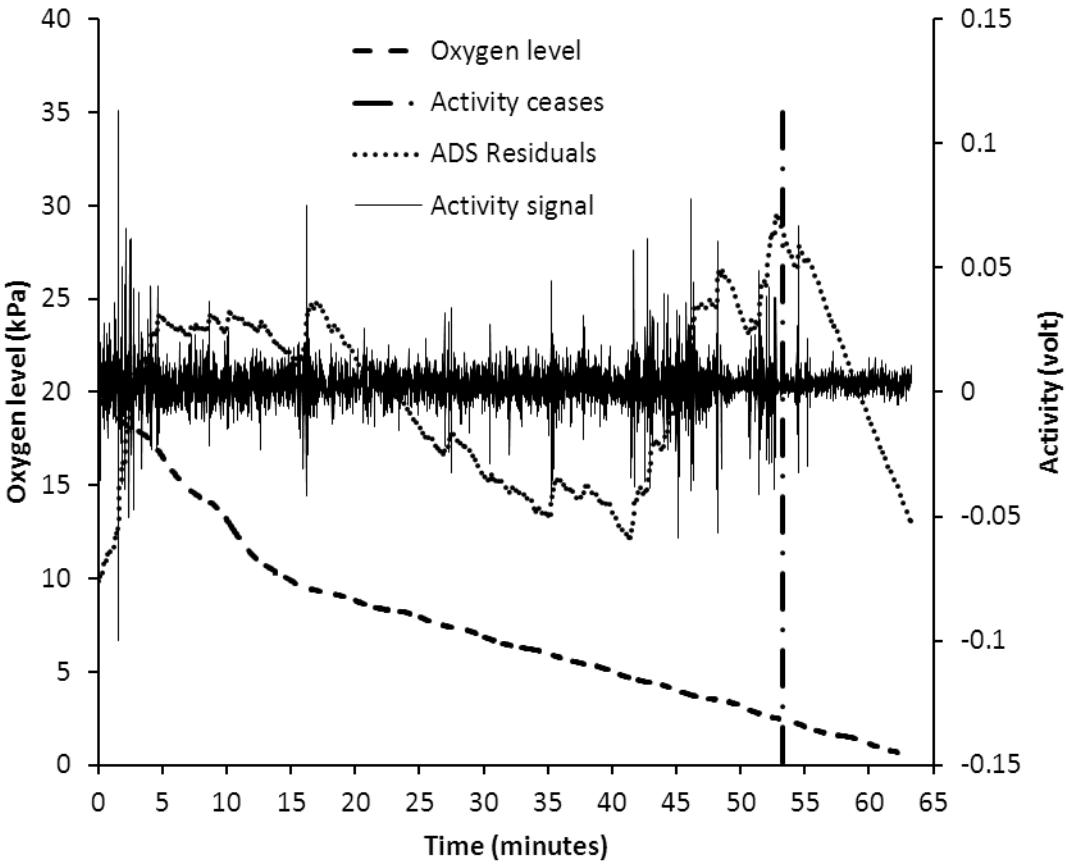

Figure 2-3: Describing larval activity in progressive hypoxia. Raw activity data were summarized by calculating the Absolute Difference Sum (ADS) for every data point: cumulative sum of absolute differences between every two previous data points up to the current data point. The residuals from a linear regression of ADS against experimental time were then calculated. Peaks in the residual plot represent decreases in activity signals, and the last peak (the vertical dash-dot line) indicates that no major activity occurs thereafter. Method based on Klok *et al.* (2010).

The larva's activity inside the chamber, including body contraction and movement of position in the chamber, was detected by the sensor as variation in the intensity of light reflected. Such data were characterized following methods used by Klok et al. (2010) (Figure 2-3). Raw activity data were in the form of voltage output from the sensor, for which the absolute difference sum (ADS) was calculated for every data point. ADS is the sum of absolute differences between all previous data points for a given point. A linear regression was calculated for the entire set of ADS against experimental time, and residuals from the regression were saved. Residuals plotted against experimental time represent the rate of change in activity data. Reduction of activity level after prolonged periods of sustained activity is represented by peaks in the residual-time plot. The time when the last peak occurs, assumed to represent activity cessation associated with the arrival of Pc, was identified from plots, and the oxygen level at the peak (referred to as activity P_C in contrast to the previous CO_2 P_C) was recorded from the raw data files.

Calculating larval oxygen consumption

Lastly, to prepare for calculations in Chapter 3, the larva's rate of oxygen consumption were calculated from the rate of CO_2 release in near normoxia. Specifically, average CO_2 production (difference of CO_2 contents in the gas flow before and after passing the chamber containing the larva)) within the one minute before oxygen level fell to 20% at the beginning of gradual hypoxia treatment was calculated. A respiratory quotient (RQ) of 1 was assumed.

$\dot{V}_{O_2} = \dot{V}_{CO_2}/RQ$, and $\dot{V}_{CO_2}$ was calculated as:

$$\dot{V}_{CO_2} = \frac{F_{CO_2} \cdot Q}{M}$$

F_{CO_2} is the average CO_2 content detected by the CO_2 analyzer during the one minute chosen, expressed as a fraction in ppm (parts per million). Assuming the scrubbed gas flowing past the larva initially contained no CO_2, having passed through CO_2-absorbing chemicals, the analyzer output would represent total production by the larva. Q is the flowrate of gas passing the sample as calibrated with the bubble flowmeter (8 mL/min), and M is the raw mass of the sample. To describe the temperature dependency of metabolism, the Q_{10} temperature coefficient (increase in metabolism when temperature is increased by 10 °C) was calculated as the ratio between the average mass-specific oxygen consumption rates at 35 °C and 25 °C. Trials for which 2 larvae were used were excluded from calculations related to mass-specific oxygen consumption.

Results

In total, 20 trials were completed in 2010 between August 10th and November 3rd, along with 15 trials in 2011 between August 10th and October 1st. Ten trials in 2010 and six trials in 2011 were conducted at 35°C, and the remaining ten in 2010 and nine in 2011 were conducted at 25°C. For most trials, only one larva with mass over 0.01g was used, but some early trials in 2010 used two larvae under the concern that CO_2 production would not be significant enough for the analyzer. Early in the 2011 season, some trials had to use two larvae again in order to reach total mass of 0.01g. All larvae that underwent the complete procedure survived. The range of air pressure at the end of trials was 97 to 100 kPa, so 1% oxygen during the stepwise decrease of oxygen level corresponded to approximately 1 kPa.

Estimating P_C from CO_2 production

Individual P_C values returned by the script varied greatly between -49.4 and 24.1 kPa, with the extreme reading being obvious irregularities. A total of 3 script results were outside the expected range (kPa equivalents of 0-20.9% oxygen), and excluding them gave a range of 0.62-18.3 kPa. Oxygen-CO_2 plots suggested not all larvae showed the standard two-part curve for which the computer program was developed (Figure 2-4), and applying the program as normal led to P_C estimations different from the visible breakpoint in the O_2-CO_2 plot (if one exists). The difference between O_2 level at the point separating two parts of the data used in the fit and O_2 level at the two regression lines' intersection was used as an indicator of the script fit's suitability, because these two values were similar in the best fits and differed in problematic fits. If the difference was over 2 kPa, then the breakpoint was visually estimated again to the nearest 1 kPa.

Five trials had to be abandoned, because three of them showed no obvious reduction ("flat" O_2-CO_2 plots) in CO_2 production between normoxia and near anoxia (although fluctuations occurred), and the other two showed greatly fluctuating CO_2 levels without a single most significant breakpoint. None of these trials used two larvae. Total CO_2 production in the three "flat" trials was low compared to other larvae tested just before and after them. Larvae used in two "flat" trials were also small (about 0.025 g) compared to other larvae at the same time of year (over 0.05 g). Low mass would not only lead to low CO_2 production mentioned above, but may also indicate poor larval health which may have affected their response to hypoxia or respiration under normoxia. A second type of non-standard plot showed a non-constant but increasing or decreasing CO_2 production before the breakpoint. P_C determined in such cases largely matched the

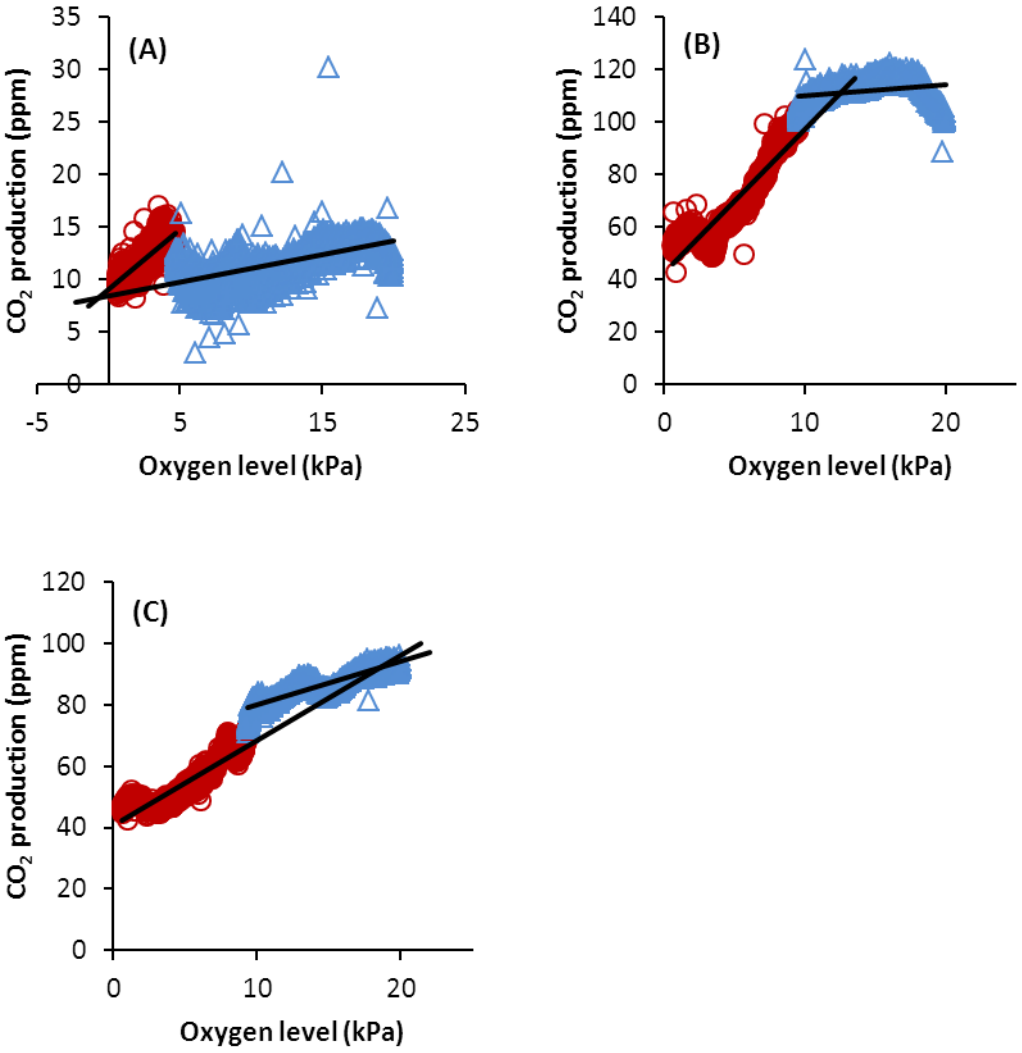

Figure 2-4: Examples of non-standard O_2-CO_2 plots. These trials likely did not satisfy the computer script's assumption of a two-part curve, and were likely to produce results (intersection of the two lines) that differed from the most significant breakpoint in the curve, if one exists. A: No overall change in CO_2 production was apparent. Although fluctuations occurred, CO_2 production at the beginning and the end of the trial was similar. B: CO_2 production did not stay entirely constant before the breakpoint. C: While CO_2 production was falling over the entire trial, a sudden fall also occurred.

visible breakpoint on the plot, and sometimes re-estimation was not required by the criteria above. A third type showed two parts during which CO_2 production falls at similar rates, separated by a sudden drop in CO_2 production. This type leads to the script returning two lines with similar slopes and intersect far away from the sudden drop. The high 18.3 kPa reading was one such trial. In this case, the sudden drop was recorded as the breakpoint. These atypical trials might have been due to imperfect habituation of the larva, leading to increases or decreases of respiration after the trial started.

In total, 15 script readings that did not match the graph were replaced with visual re-estimations, with an average of 9.9 kPa, standard deviation of 0.6 kPa and range of 8-11 kPa. The remaining 15 acceptable script results averaged 9.3 ± 3 kPa, with range of 4.4-12.7 kPa. The properly fitted script results and visually re-estimated results also did not differ significantly from each other (two-tailed t-test: $t = 0.74$, $p = 0.47$). Together all 30 results averaged 9.5 ± 2.2 kPa. The script and visual results, either analyzed together or separately, showed no influence from temperature treatment or year (Table 2-1). No consistent change over time was observed either (Figure 2-5).

Activity cessation in progressive hypoxia

There were 7 trials during which activity was increasing at the end of the progressive hypoxia, and therefore activity cessation likely took place later in near anoxia. Among the rest of the trials, the last major decrease in activity (peak in ADS residual – time plots) occurred at on average 3.6 ± 2.4 kPa. The extreme values were 10.3 – 0.8 kPa. Some trials could be seen showing very little activity compared to others made in similar times on similarly-sized larva. Although robust mathematical comparison

Table 2-1: Testing for differences of CO_2 P_C between years and temperatures[a]

A. CO_2 P_C, testing all data (n=30)

Source of variation	df	Sum of squares	F	p[b]
(Intercept)	1	5.04	1.14	0.296
Temperature	1	4.42	1.00	0.327
Year	1	5.03	1.14	0.296
Interaction	1	4.42	1.00	0.327
Residuals	26	115.23		

B. CO_2 P_C , script data only (n=15)

Source of variation	df	Sum of squares	F	p
(Intercept)	1	10.13	1.13	0.311
Temperature	1	9.45	1.05	0.328
Year	1	10.13	1.13	0.312
Interaction	1	9.45	1.05	0.327
Residuals	11	98.95		

C. CO_2 P_C, visual data only (n=15)

Source of variation	df	Sum of squares	F	p
(Intercept)	1	0.58	1.94	0.191
Temperature	1	0.56	1.89	0.197
Year	1	0.58	1.93	0.192
Interaction	1	0.56	1.89	0.197
Residuals	11	3.28		

[a] Tested with type III SS, in R 2.12.0 with the Anova() function in the *car* package.

[b] No statistical significance was detected at p = 0.05.

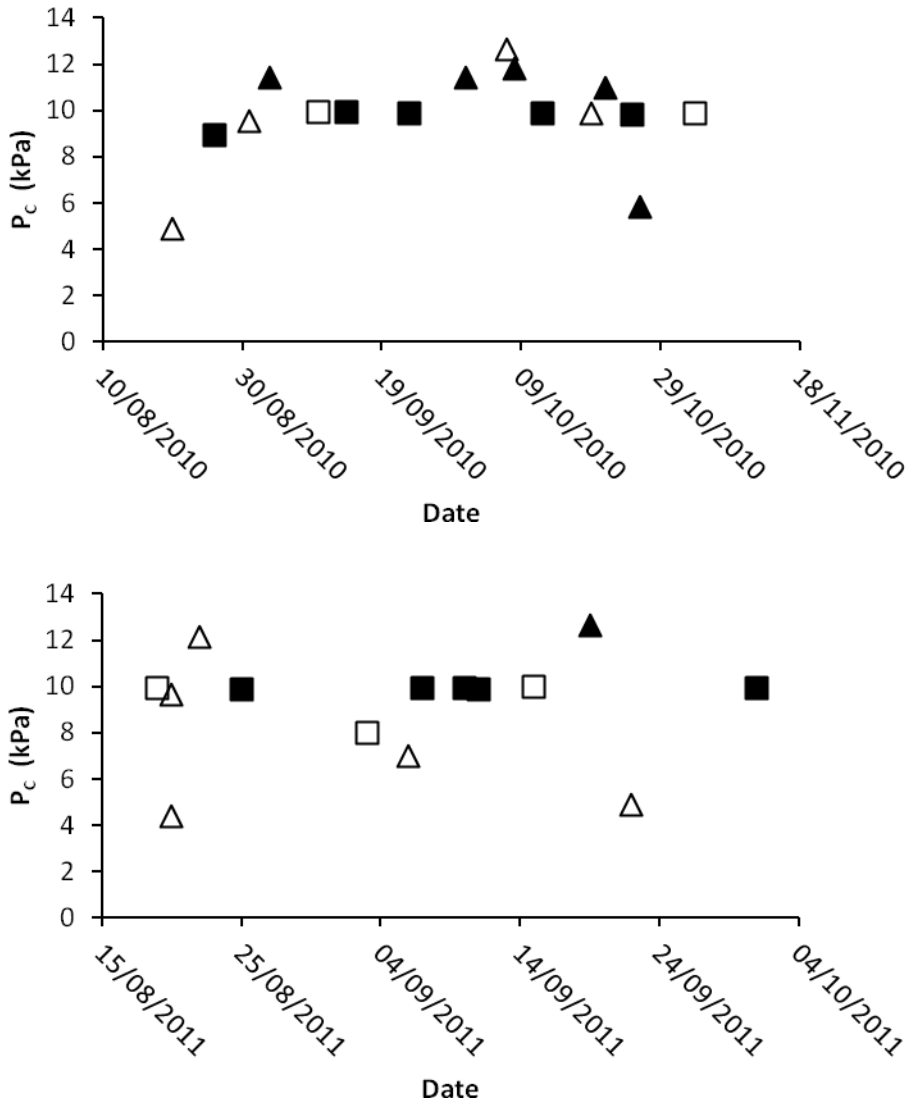

Figure 2-5: CO_2 P_C values over the growth season. No obvious trend over time was identified. Triangles represent script-fitted results that fitted the O_2-CO_2 curve, and squares represent results from visual re-estimation. Empty markers represent trials at 25°C, and filled markers represent trials at 35°C.

between trials was impossible given the lack of calibration in raw voltage data, we still attempted to quantify the amount of activity signal against voltage fluctuations using the ratio of range:mean in raw activity data. The trials visually assessed as having low activity had ratios of below 14, while the same ratio in other trials generally fell above 30 and 300 with the maximum at 636. Subsequently, all trials with the range:mean ratio below 14 were deemed "low activity" trials, including two trials not initially identified as such. In the end, fifteen trials showed low activity that could have simply represented random signal fluctuations in contrast to 20 with higher activity levels. Among the 7 trials during which activity was increasing at the end, 5 showed low activity. In the remaining two such trials, some activity persisted after the gas supply has been switched to nitrogen, and oxygen level stopped falling (Figure 2-6B). After excluding these 7 trials (because no obvious point of activity cessation could be identified), activity P_C did not differ significantly (two-tailed t-test: $t = 0.36$, $p = 0.72$) between the high- (3.7 ± 2.4 kPa) and low-activity (3.4 ± 2.3 kPa) trials. Activity P_C was not well-correlated with CO_2 P_C (Pearson's $r = 0.21$, $p = 0.32$), had no obvious trend over the season (Figure 2-7) and showed no significant influence by year or temperature (Table 2-2).

Larval oxygen consumption in normoxia

The larva's mass-specific oxygen consumption rates ($\dot{V}_{O_2}$) were affected by temperature, with Q_{10} of 1.46 in 2010 and 1.55 in 2011, and 1.46 when data from both years were used (Figure 2-8). Larval mass did not differ significantly between temperature treatments (excluding 2-larvae trials, two-tailed t-tests: 2010 $t = 0.34$, $p = 0.75$; 2011 $t = 0.016$, $p = 0.99$; both years $t = 0.33$, $p = 0.75$). Therefore, Q_{10} values should not be confounded by mass differences between temperature treatments. However,

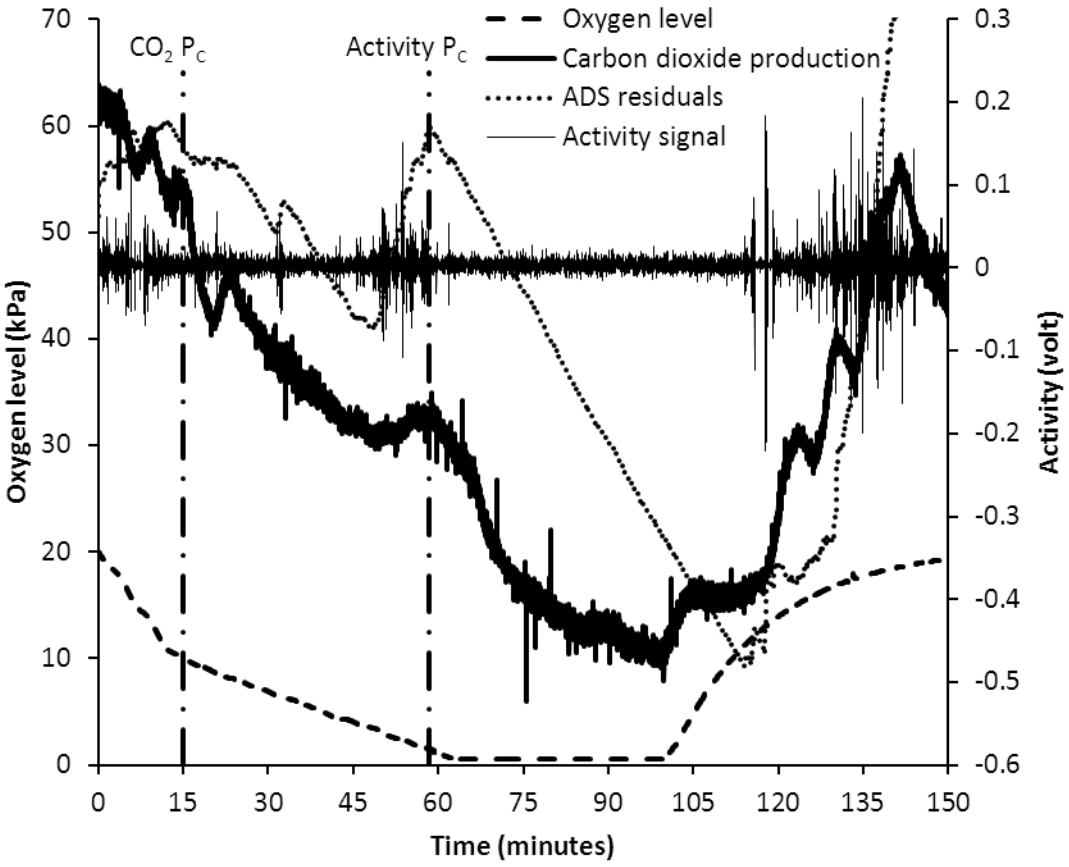

Figure 2-6(A): Common activity patterns of *E. solidaginis* larvae in progressive hypoxia. Here, a period of low activity occurred between the CO_2 and activity P_Cs. The initial decrease may represent one way by which the larva's metabolic rate is reduced, and elevated activity before the activity P_C may represent exploratory or escape response under severe hypoxia. The reduced activity level after the activity P_C persisted until oxygen supply was restored.

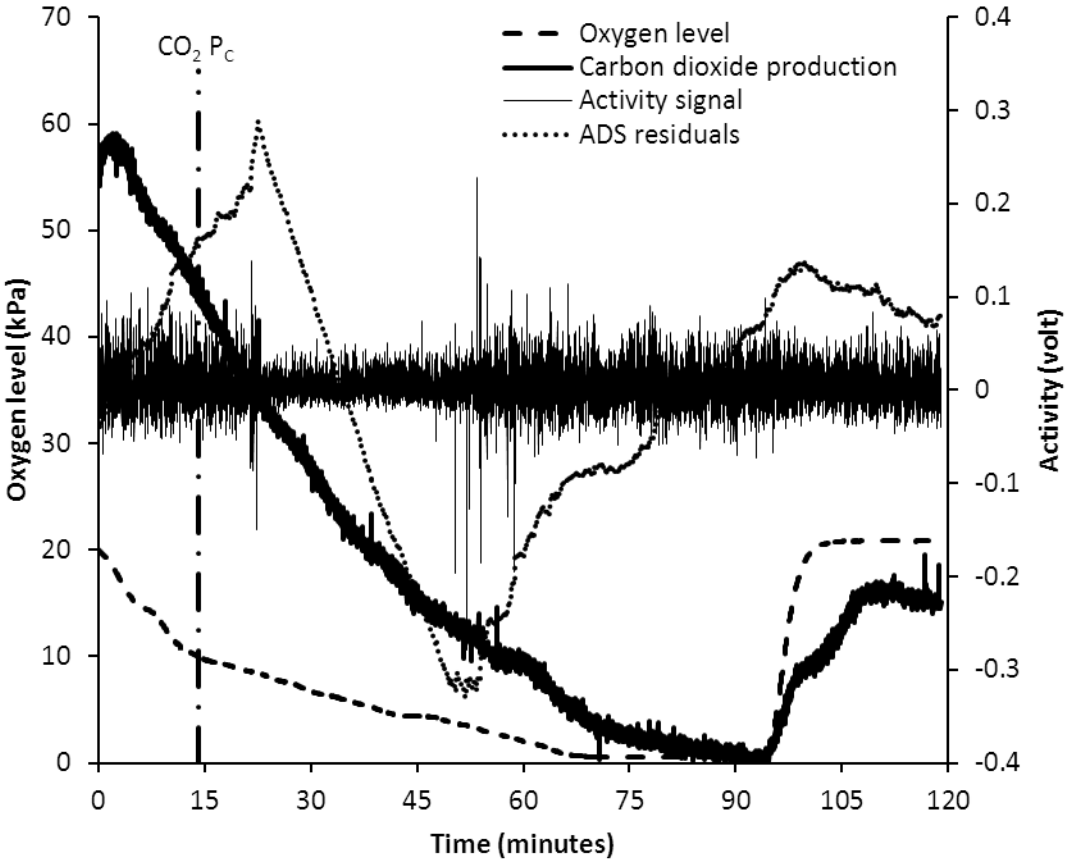

Figure 2-6(B): Patterns in 2-6A were not uniform among all trials. The period of low activity may start at an oxygen level below the CO_2 P_C, suggesting metabolic depression at the CO_2 P_C was not entirely a result of reduced activity. After exhibiting high activity in severe hypoxia, low activity level was sustained through the 30 minutes of nitrogen exposure, so the activity P_C was not identified during the exposure to progressive hypoxia. There was no obvious increase of activity after normoxia was restored, despite increasing CO_2 production.

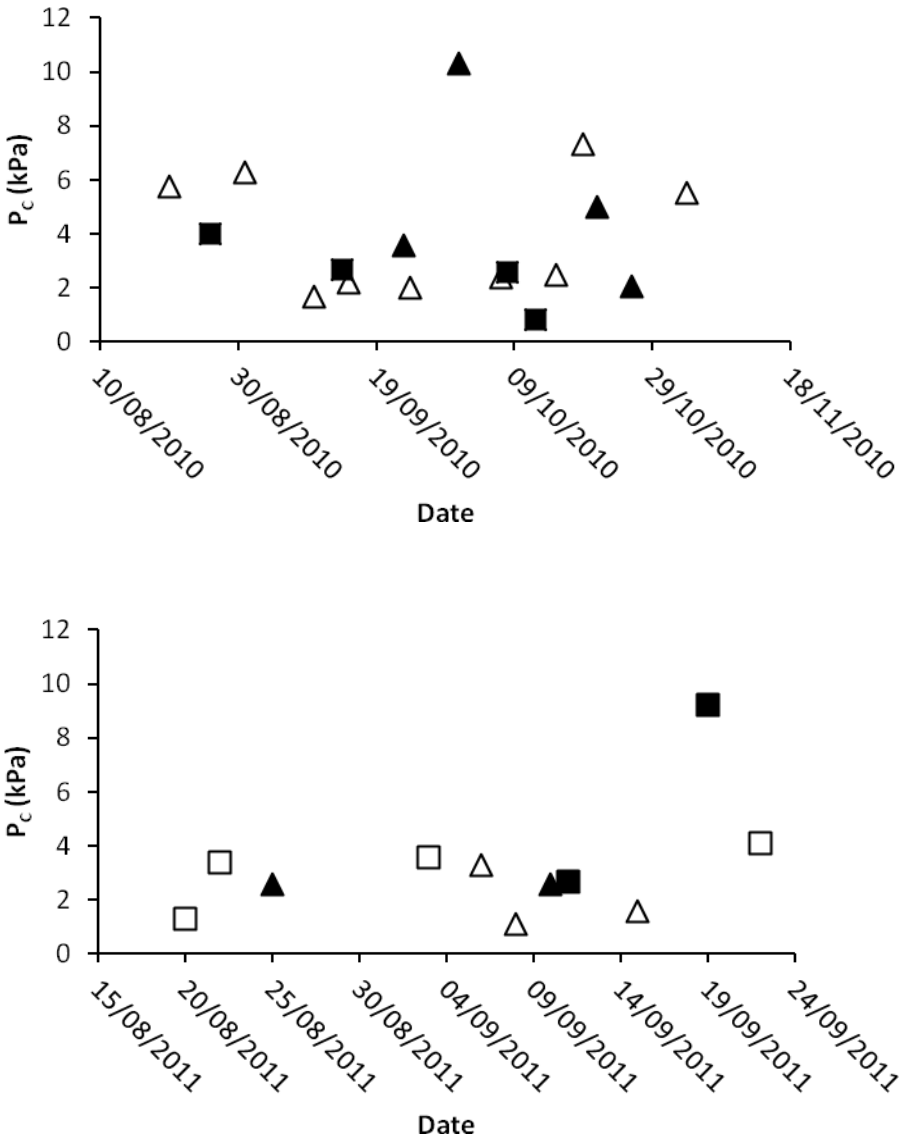

Figure 2-7: Activity P_C values over the growth season. As in CO_2 P_C, activity P_C showed no obvious trend over time either. Squares represent trials with low activity (indicated by range:mean ratio), and triangles represent trials that showed higher activity. Empty markers represent trials under 25°C, and filled markers represent trials under 35°C.

Table 2-2: Testing for differences of activity P_C between years and temperatures[a]

A. Activity P_C, all data (n = 28)

Source of variation	df	Sum of squares	F	p[b]
(Intercept)	1	5.86	1.01	0.326
Temperature	1	4.73	0.81	0.376
Year	1	5.85	1.01	0.326
Interaction	1	4.73	0.81	0.376
Residuals	24	139.58		

B. Activity P_C, high activity trials only (n = 18)[c]

Source of variation	df	Sum of squares	F	p
(Intercept)	1	0.005	0.001	0.978
Temperature	1	0.406	0.071	0.794
Year	1	0.005	0.001	0.978
Interaction	1	0.405	0.071	0.795
Residuals	14	80.45		

[a] Tested with type III SS, in R 2.12.0 with the Anova() function in the *car* package.

[b] No statistical significance was detected at p = 0.05.

[c] Could not test low activity trials as two-way ANOVA because no 25°C trials in 2010 showed low activity. Testing as one-way ANOVA by combining year and temperature as one factor showed no significant difference (F = 1.862, p = 0.225).

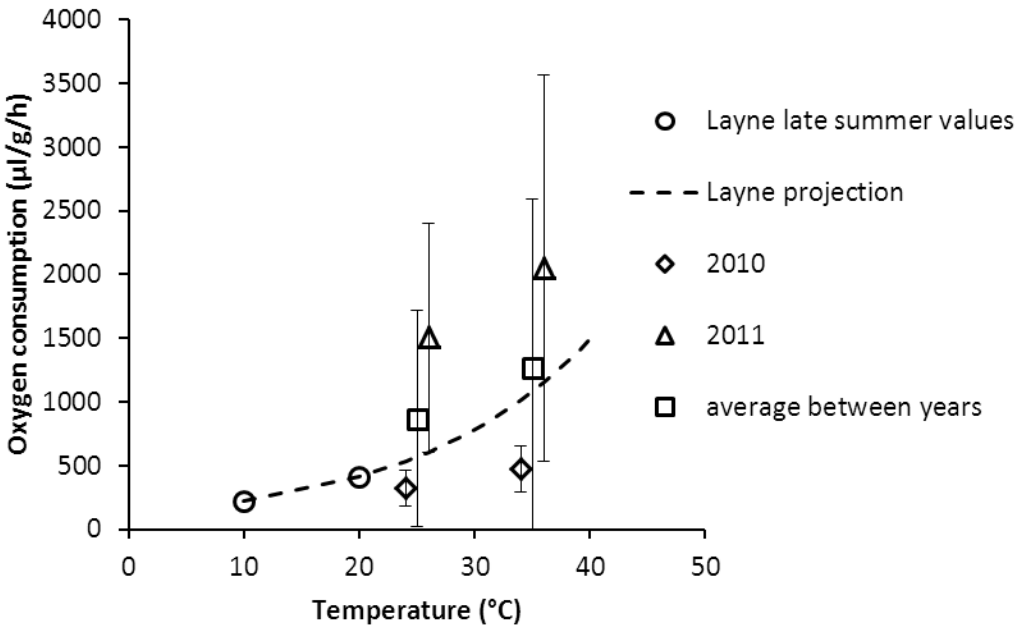

Figure 2-8: Temperature-dependent metabolism of *E. solidaginis* larvae. Error bars represent standard deviation. Oxygen consumption was calculated from CO_2 production assuming RQ of 1. Late summer metabolic rates (and projection to higher temperatures via Q_{10}) estimated by Layne and Eyck (1996) were plotted for comparison. Symbols are plotted with minor horizontal offset (1°C) to avoid overlapping error bars.

average $\dot{V}_{O_2}$ from year 2011 more than doubled with regard to 2010 results at the same temperature. Larvae in 2010 had significantly higher mass (one-tailed t-test: $t = 2.88$, $p = 0.004$, 2-larvae trials excluded). $\dot{V}_{O_2}$ generally showed negative correlation with body mass. The correlation between total CO_2 production and mass, as well as the extent of allometric metabolic scaling, was highly variable and inconsistent between groups of data (Figure 2-9, Table 2-3).

Potential difference in P_C due to sample source and experiment delay

In total, 9 trials used larvae from outside Area B, and larvae in 7 of them were extracted at an earlier date. Among the 9, 3 trials were among those abandoned from CO_2 P_C trials, and another was abandoned from activity P_C estimation. Two CO_2 P_Cs could be considered outliers (having values beyond the range of mean ± 2 standard deviations) from Area B data collected in the same year under the same temperature treatment. One of these two also had activity P_C being an outlier. The 9 trials did not consistently or exclusively show low activity or the need for visual estimation of CO_2 P_C. Analyzing only data from Area B larvae did not lead to greater significance in ANOVAs regarding differences in P_C between temperature and years. The resultant difference in the overall mean P_Cs was less than 0.5 kPa (Area B only: activity P_C 3.2 ± 1.8 kPa, CO_2 P_C 9.4 ± 2.1 kPa), and the difference in standard deviation was less than 1 kPa. Therefore, subsequent discussion would be based on data collected using larvae from all locations (all-inclusive: activity P_C 3.6 ± 1.4 kPa, CO_2 P_C 9.5 ± 2.2 kPa). The effect on Q_{10} was similarly low (Area B only: 2010 $Q_{10} = 1.36$, 2011 $Q_{10} = 1.56$).

Discussion

Discrepancy between metabolic depression and activity cessation

Oxygen levels at which abrupt decreases in CO_2 production and activity level occurred were similar in *Drosophila melanogaster* (Klok *et al.*, 2010), but that was not the case in our *E. solidaginis* data. Is it possible that CO_2 P_C and activity P_C represented distinct physiological processes? As discussed in Pörtner and Grieshaber (1993), metabolic depression that occurs in hypoxia (oxyconformity) may be divided into two types: Anaerobic oxyconformity occurs when oxygen supply to mitochondria is insufficient. The critical point for anaerobic oxyconformity is known as Pc_M below which anaerobiosis starts. Aerobic oxyconformity on the other hand, describes the situation when anaerobiosis is not necessary, but oxygen consumption still decreases with falling oxygen level. The onset of aerobic oxyconformity is described by Pc_R which is higher than Pc_M. If this distinction applies to our data, the higher CO_2 P_C would represent Pc_R, and activity P_C would represent Pc_M. However, alternative oxidases suggested as the basis for aerobic oxyconformity (Pörtner, 2010) have not been found in insects, including four representatives from Diptera and seven others from Coleoptera, Hemiptera, Hymenoptera, Lepidoptera and Phthiraptera (McDonald *et al.*, 2009). Discussion of aerobic oxyconformity mainly pertained to marine invertebrates (Pörtner and Grieshaber, 1993). Therefore, it is more likely that the inconsistency should be explained by reasons other than aerobic oxyconformity.

Determination of the true Pc_M depends on estimates of the animal's standard metabolic rate when all organs are at rest (Pörtner and Grieshaber, 1993), and animals

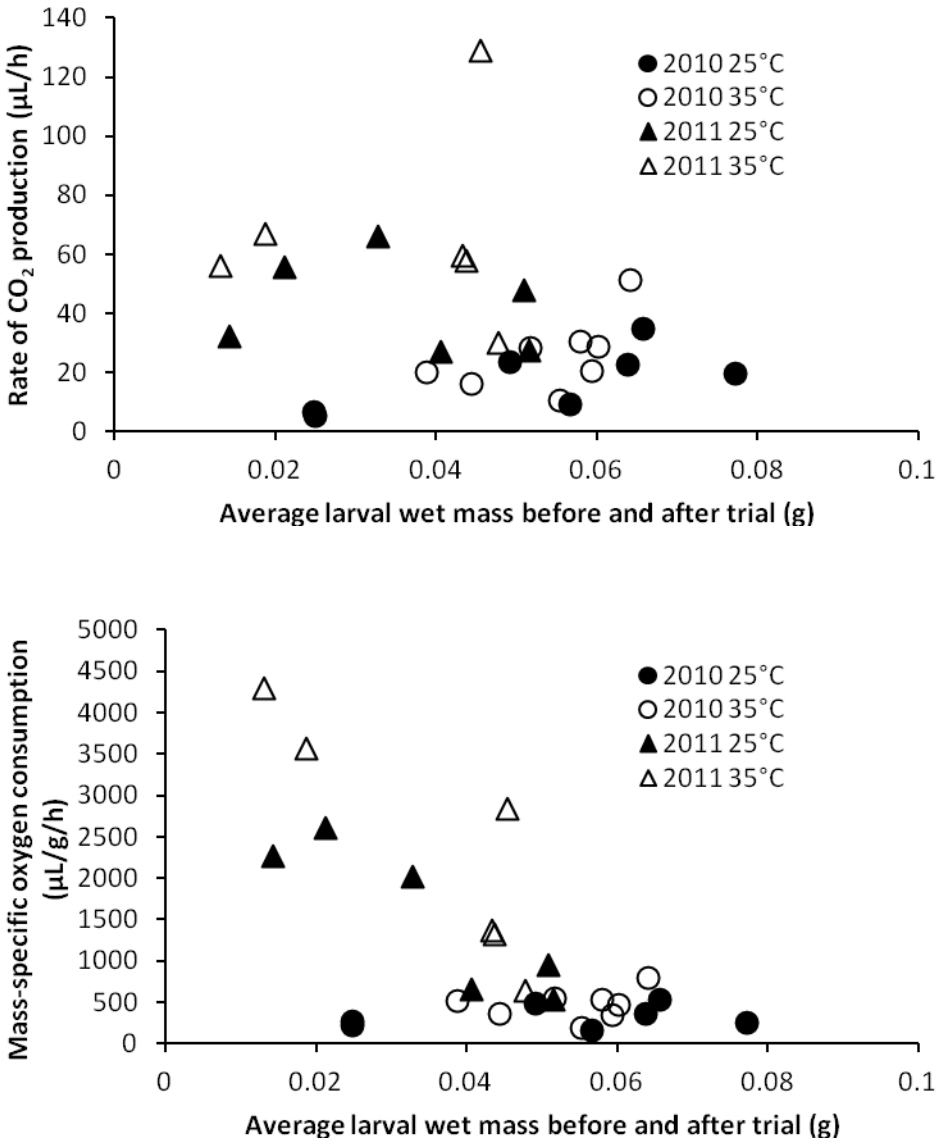

Figure 2-9: Correlation between metabolism and body mass in *E. solidaginis* larvae. Total CO_2 production at normoxia fell within similar ranges and did not significantly correlate with body mass (see Table 2-3). Mass-specific oxygen consumption showed negative correlation with mass (also see Table 2-3). Larvae used in 2010 were also significantly larger despite having been taken from the same sites throughout the growth season.

Table 2-3: Correlation between metabolism and larval mass.

Correlation of larval mass[a] with:	Total CO_2 production[b]		Mass-specific O_2 consumption rate ($\mu L/g/h$)[c]		Mass-metabolism allometry[f]
	r[d]	p[e]	r	p	
2010 25°C	0.697	0.082 n.s.	0.280	0.543	1.27
2010 35°C	0.561	0.148	0.246	0.558	1.26
2011 25°C	-0.222	0.678	-0.897	0.015*	-0.11
2011 35°C	0.109	0.838	-0.873	0.023*	-0.01
All 2010	0.568	0.027*	0.241	0.387	1.36
All 2011	0.002	0.995	-0.801	0.002**	-0.06
All 25°C	-0.164	0.593	-0.608	0.028*	0.05
All 35°C	-0.323	0.260	-0.835	< 0.001**	-0.55
All data	-0.221	0.267	-0.684	< 0.001**	-0.17

[a] Average wet mass before and after the trial

[b] Rate of CO_2 production by entire sample ($\mu L/h$)

[c] Calculated assuming RQ of 1.

[d] Pearson's correlation coefficient, calculated in R 2.12.2.

[e] n.s. $p < 0.1$, * $p < 0.05$, ** $p < 0.01$

[f] Regression coefficient of $\log_{10}$(total CO_2 production) against $\log_{10}$(mass)

maintaining intensive activity such as flight will exhibit higher P_C (Harrison and Lighton, 1998). If *E. solidaginis* larvae in the experiments initially maintained high activity, they could have shown an elevated P_C that represented a reduction of metabolic rate when the high activity level could no longer be maintained. Because raw activity readings were influenced by uncontrolled factors such as the larva's size and position over the activity sensor, the magnitude of activity readings could not be compared directly between trials. However, examination of the activity plots did not find consistently high activity followed by a fall in activity levels at the CO_2 P_C. Among the 10 trials with CO_2 P_C of at least 10 kPa (script and visual estimates) and a strong activity signal (range:mean ratio > 14), 8 showed obvious activity at oxygen levels above the CO_2 P_C, but only 2 among them showed decreasing activity shortly after oxygen level fell below the CO_2 P_C (as shown in Figure 2-6A). Otherwise, similar levels of activity may be maintained at oxygen levels above and below the CO_2 P_C (Figure 2-6B). *E. solidaginis* larvae are not very mobile, so it is possible that their activity does not require intensive aerobic metabolism, and therefore could be maintained even with reduced metabolism. This however, means the larva reduced its metabolic rate through processes other than physical activity, such as digestion and growth.

It is also possible that the decrease in CO_2 production is not conforming to the oxygen level directly. If a metabolic depression is triggered by hypoxia below a certain oxygen level and develops gradually through time, then the progressively more severe hypoxia will appear correlated with the decreasing metabolic rate. Metabolic depression is a common response to hypoxia (Harrison *et al.,* 2006). During winter diapause, *E. solidaginis* is capable of significant metabolic depression (Irwin *et al.,* 2001) and also

acquires tolerance to freeze-imposed tissue hypoxia (Morin *et al.*, 2005), so the metabolic depression may in part be a result of the animal's response to hypoxia (in addition to, for example, temperature dependency of metabolism). It is likely that at least some physiological processes used for hypoxia tolerance during the winter can also be used when facing hypoxia not associated with winter conditions. How the process is regulated under summer and autumn temperatures while maintaining activity, as opposed to under low winter temperatures while remaining inactive (or even frozen), may be a point of interest for future studies.

Metabolic depression in oxygen levels well above the onset of anaerobiosis may be relevant to the larva's lifestyle. Klok *et al.* (2010) recorded elevated activity levels in *D. melanogaster* adults and larvae when oxygen level fell to 6-7 kPa, reaching maximum activity between 2-4 kPa, possibly demonstrating exploratory or escape response. Such behaviour facilitates the search for less hypoxic habitats for free-living animals. However, the escape response is unlikely to be adaptive for *E. solidaginis* larvae. Moving outside the gall is impossible before excavation of the exit tunnel, and also detrimental due to the loss of food source and protection. Although the chamber initially starts as an elongated tunnel, it eventually expands into an approximately spherical gas-filled space. If hypoxia occurs in the chamber, it is unlikely that a significant oxygen gradient will develop, so movement within the chamber is unlikely to take the larva away from hypoxia. In this situation, the ancestral (if present in a free-living ancestor) escape behavior from hypoxia is not only ineffective in facilitating escape, but also represents a waste of energy due to increased energy cost from elevated activity level. Therefore, the escape behavior should be selected against by the confined habitat, and metabolic

depression that conserves energy may be more adaptive for *E. solidaginis* during hypoxia. Some animals did exhibit an increase of activity level before the final activity cessation (Figure 2-6), demonstrating that the escape behavior is not completely lost. On the other hand, the higher CO_2 P_C may suggest the larva is sensitive to hypoxia, and even relative mild hypoxia that does not require anaerobic metabolism may trigger a regulated metabolic depression.

Explanations above assume that the lower activity P_C represents a better estimate of Pc_M, but the reverse could also be true. Certain dipteran larvae that feed on decaying matter cannot survive anoxia indefinitely (maximum survival time 4 hours at room temperature, von Brand, 1946), so the ability to sustain activity in severe hypoxia or even anoxia is important for escaping hypoxia (Hoback and Stanley, 2001). In *D. melanogaster* larvae, such activity is likely sustained by anaerobic energy production (Klok *et al.*, 2010). Therefore by definition activity is maintained below Pc_M, and the cessation of activity thereafter does not indicate Pc_M. If activity is not maintained indefinitely under such conditions, the final cessation of activity could be identified as the P_C if it is more significant than other changes in activity level. In Klok *et al.* (2010), elevated activity level in oxygen levels approaching the P_C enabled the identification of a significant breakpoint in the ADS residuals. In the case of *E. solidaginis* however, a weakened or non-existent escape response and low activity level in general (as a result of its confined lifestyle) could mean there is only weak change of activity level at the onset of anaerobiosis, and the eventual activity cessation after a period of anaerobic activity would be identified as the P_C. Sustained low activity (above the lowest level recorded during the trial) through the 30-minute nitrogen exposure was indeed observed for *E.*

solidaginis larvae in some trials (Figure 2-6B). However, further decrease of activity in this situation occurred during the nitrogen exposure, and therefore would not be identified at a particular oxygen level during the progressive hypoxia treatment. Alternatively, the apparent activity during the nitrogen exposure could be caused by lower activity signals that occurred before nitrogen exposure started. While the period of low signal could represent inactivity by the larva, they could also be affected by the animal's movement relative to the sensor. A previous period of low activity signal (whether truly representing low activity or not) could have affected the regression, so that stronger signals due to location during the nitrogen exposure would be interpreted as a period of high activity. In this case, the capacity for sustained activity in anoxia is not supported, which is consistent with the larva's inability to escape the gall habitat, and still suggests that the activity P_C is a more likely estimate for Pc_M.

While determining the exact nature of the CO_2 and activity P_Cs may require further study (possibly involving detecting the onset of anaerobic metabolism), they still provide points of reference for discussion of environmental hypoxia in subsequent chapters. CO_2 P_C represents the oxygen level at which metabolic depression starts, whether as an oxygen-limited process or as a regulated physiological process used by the larva to conserve energy. The metabolic depression would put the larva at a disadvantage in feeding and growth, which requires physical activity and elevated metabolism. Meanwhile, elevated activity levels are not severely inhibited, so feeding may still take place. Activity P_C on the other hand represents the oxygen level below which the larva exhibits very low activity levels, leading to severe depression of feeding activity and growth.

Other potential influences on P_C

As insects mature, they may develop better gas exchange capacities and therefore reduce their P_C in later development stages (Greenlee and Harrison, 2004). The increase in tracheal diameter, an important contribution to gas exchange capacity, only takes place shortly after ecdysis when the old cuticle lining the trachea has been removed and the new layer has yet to harden (Beitel and Krasnow, 2000). Without ecdysis, the increase in O_2 delivery capacity may be insufficient to support the increase of body mass within an instar, leading to increased P_C and possibly necessitating ecdysis (Greenlee & Harrison 2005). Mealworm (*Tenebrio molitor*) larvae under hypoxia molted more frequently, presumably due to greater need to improve gas exchange (Greenberg and Ar, 1996). Of course, the free-living mealworm larva has a tough cuticle, unlike *E. solidaginis* larvae which are protected by the gall and have a more flexible cuticle. A better comparison with *E. solidaginis* may be the more closely related and similarly soft-bodied *Drosophila melanogaster* larvae, which increase their tracheal diameter at ecdysis (Beitel and Krasnow, 2000). Therefore it is likely that *E. solidaginis* development involves a similar process.

However, a consistent change in P_C with time was not observed in our experiment (Figure 2-5). *E. solidaginis* undergoes three instars as larva. Ecdysis typically takes place in late July and early August, with the first two instars lasting 4 and 3 weeks respectively (Uhler, 1951). After that, there is no more opportunity to increase tracheal diameter until pupation and emergence. Trials started on August 10th in both years, so larvae that hatched more than seven weeks earlier (June 22nd) were likely in the third instar. Hatching takes place over 17-18 days (Uhler, 1951). In this study, oviposition was first

observed on May 26th in 2010 and June 1st in 2011, 27 and 21 days before June 22nd respectively (see Chapter 4 for further discussion on phenology). So the vast majority of all larvae used were likely already in the third instar, and variation in P_C cannot be attributed to tracheal remodelling at ecdysis. The larva's body length (Uhler, 1951) and mass (Layne and Eyck, 1996) may more than double within the third instar. Ventilation and tracheal branching are apparently sufficient to support such growth, perhaps in part due to the larva's low activity lifestyle in a confined space. Trachea remodelling is still possible before emergence from the pupa, and the adult likely needs better adaptations for temporarily elevated metabolism since it is capable of flight.

Temperature was another factor expected to influence P_C, but the influence was not observed in our study either. Temperature dependence of metabolic rate was observed, so a temperature-dependent P_C should be possible because elevated metabolic rate due to high temperature would be more difficult to maintain at a given oxygen level. The effect could have been confounded due to other influences, since responses to progressive hypoxia, in terms of CO_2 production and activity patterns, were inconsistent. Such inconsistency may be related to the habituation process. As seen in some of the O_2-CO_2 curves, sometimes the larva's CO_2 production did not remain constant before the most obvious breakpoint in the curve (Figure 2-4 B, C). This may indicate that the larva did not fully habituate to the new environment during the 15 minutes given for habituation. Being adapted for a confined habitat surrounded by moist plant tissue, it may have been difficult for the larva to habituate to the open space (depending on body size) in the respirometer surrounded by glass. This may have interfered with the larva's normal response to progressive hypoxia, regardless of what the normal response may be, leading

to inconsistency of activity and metabolism patterns. In preparation for similar experiments in the future, it may be useful to find a way to evaluate the larva's habituation, or design a chamber somehow resembling the gall habitat.

The source of larvae used was also considered as a potential influence. In contrast to the open field at Area A (Figure 1-3), host plants in Area B mostly occurred along tree fences and forest areas which should provide more shading to the gall. Difference in shading due to plant cover may influence gall temperature, which is affected by direct sunlight (Layne, 1993). Larvae adapted to these locations could show different physiological adaptations to extreme temperature that may influence metabolism and P_C. Furthermore, the delay between larva extraction and the experiment could have led to more variation in larval health. The presence of outliers in Area A data relative to Area B means and standard deviations could be evidence for such effects, but the small number and effect of the outliers on the overall mean and standard deviation might indicate that other sources of variation were more important than host location effects. One last potential influence on larval health unaccounted for during the study was parasitoid infestation. The parasitoid wasp *Eurytoma obtusiventris* lays its eggs inside *E. solidaginis* larvae, and causes premature pupation in the host larva. Given the parasitoid's influence on host physiology, it is possible that infested *E. solidaginis* larvae would show different metabolism and/or activity than healthy larvae.

How P_C may reflect habitat requirement

Among insects, P_C values of *E. solidaginis* larvae were not among the lowest. P_C estimated via O_2 consumption were lower in some other insect species (4.6-7.6 kPa in

Proischnura subfurcatum, Apodaca and Chapman, 2004; 4-8 kPa in *Manduca sexta* larvae, Greenlee and Harrison, 2005; 2-6 kPa in *Aphodius, Geotrupes, Sphaeridium* spp., Holter and Spangenberg, 1997), while the activity P_C had a more similar range to the above species. Meanwhile, *D. melanogaster* larvae exhibit a lower activity P_C of about 1 kPa, even without considering the larva's sustained activity in near anoxia (Klok *et al.*, 2010). Similarly, *Orthosoma brunneum* larvae maintained normal activity levels until oxygen level fell below 1% (about 1 kPa), and merely appeared sluggish in 0.8-0.9% O_2 (Paim and Beckel, 1964). High P_C does not necessarily indicate the insect never encounters hypoxia below its P_C in nature. For example, *Phormia regina* larvae which feed on carrion (Joy *et al.*, 2006), a potentially hypoxic habitat, showed relatively high P_Cs in the range of 10-15% oxygen (Keister and Buck, 1961). These species may move within their habitat to avoid severely hypoxic areas (Hoback and Stanley, 2001). Others active in open air, such as adult grasshoppers, may have P_C below 2 kPa (Greenlee and Harrison, 2004). Their low P_C may reflect not habitat requirements but other respiratory adjustments, such as the requirement for high gas exchange efficiency to support increased body size and intense activities such as flight. At higher instars, they gain better control over ventilation rates and larger tidal volume (Greenlee and Harrison, 2004). Tracheal diameter and/or branching (depending on species) also increase in response to hypoxia during development (Harrison *et al.*, 2006). These adaptations would confer better tolerance to hypoxia in experiments, and may also be useful if the insect experiences hypoxia in nature, such as at high altitude or in burrows. Conversely, nymphs have not yet developed these adaptations and have high P_C which decreases during development (Greenlee and Harrison, 2004). However, as seen in species that exhibit low

P_C as larvae (Klok *et al.,* 2010; Paim and Beckel, 1964), developmental constraints should not preclude insect larvae from evolving very low P_C.

As discussed above, the larva is likely unable to leave the gall or move within the chamber in order to escape potential hypoxia. Expanding the chamber may reduce the diffusion distance for respiratory gases or at least create a gradient in oxygen level, but the larva does not dig outside the nutritive layer until exit tunnel excavation in late autumn. By then, the dying plant would compete less for oxygen, and larval growth would also be slowing down, so contribution of the exit tunnel to the larva's oxygen requirement is likely minimal. Without modifying larval behaviour, one possibility to reduce potential hypoxia via gall modification is expanding the nutritive layer so that the chamber eventually expands to a greater diameter and has a thinner wall. However, the effectiveness of such modification would be limited by how fast the larva consumes nutritive tissue. In addition, thickness of the nutritive layer shows little heritable variation in the first place (Weis *et al.,* 1989). The nutritive layer represents a significant energy cost to the host plant, so its size may be limited by the degree to which the larva may redirect host resource without increasing the selection pressure for host resistance against the gallmaker, which may explain why heritable variation in gall size mainly comes from the less metabolically active and less costly cortex layer (Weis *et al.,* 1989). Under such selection, nutritive layer thickness could have reached its optimum so that little variation remains. With no means to escape gall hypoxia (if it occurs), *E. solidaginis* larvae should be under selection for a P_C that enables a stable metabolism at oxygen levels prevalent in the gall.

Temperature and mass dependencies of metabolic rates

E. solidaginis galls have no insulating properties. Temperature inside the gall largely follows air temperature, but may rise higher under direct sunlight (Layne, 1993). Therefore gall temperature should be affected by factors that influence air temperature and sunlight exposure, which include time of day, weather, season and location. The larva's temperature dependency of metabolism from late summer to early winter was previously observed in Layne and Eyck (1996). And even in diapause with severely depressed metabolism, energy expenditure by *E. solidaginis* is still affected by temperature (Irwin and Lee, 2003). In addition, host plant tissue metabolism also exhibits temperature dependency (see Chapter 3). So if hypoxia occurs in galls, temperature will likely affect its severity, due to the effects of temperature on the rate of oxygen consumption by gallmakers and hosts alike.

Average mass-specific oxygen consumption by *E. solidaginis* larvae were similar to values extrapolated from late summer (6-11 September) data and Q_{10} estimated by Layne and Eyck (1996), and the Q_{10} exhibited in 2010 (1.4) and 2011 (1.6) fell within the seasonal variation (1.4 - 1.9) estimated in the same study. Mass-specific metabolism is expected to correlate negatively with mass, due to the negative allometry of total metabolism. This effect was apparent in Layne and Eyck (1996), specifically in results from late summer and autumn when larval mass doubled. Besides the steeply negative allometry of mass-specific metabolism, acclimatization to seasonal temperature and reduction of growth rate in late autumn were suggested as influences on metabolic rate by Layne and Eyck (1996). Since P_C trials started nearly a month earlier (August 10th) than earliest measurements by Layne and Eyck (Sep. 6^{th}), seasonal difference would be less

apparent over the entire sampling period, so acclimatization may not be as significant an influence.

Significant difference in larval mass between year 2010 and 2011 may have added to the effect of allometric scaling, and may therefore explain the large difference in mass-specific oxygen consumption between years. However, the extent of scaling was not only different from the level expected by Kleiber's law (0.75), but also showed inconsistency between groups of data. It is likely that confounding factors were present in at least some data groups. Since oxygen consumption was measured at the beginning of each progressive hypoxia trial, incomplete habituation as discussed above could be involved. The difference of body mass between years may also indicate different health conditions, which should affect larval metabolism. Lastly, larvae used for respiration measurements differed in the time of collection and age (indicated by different gall appearance dates, detailed discussion in Chapter 4) between the two years. Although the larvae were unlikely to be in different instars as discussed earlier, if metabolism is affected by age and/or development through mechanisms other than mass dependency, the results would be affected. For calculations in Chapter 3, only the average mass-specific metabolic rates were used, and detailed relation between mass and metabolic rate was not taken into account. Differences in larval health and environmental conditions between the two years are discussed further in Chapter 4.

Chapter summary

E. solidaginis larvae exhibited metabolic rate and activity depressions at different oxygen levels, possibly due to regulated metabolic depression or activity being sustained

by anaerobiosis. The oxygen level at which these depressions occur suggested that the larva's tolerance to hypoxia was not exceptionally good among insects. Because the animal does not exit the gall until its emergence as an adult, larval physiology should be adapted to conditions inside the gall. Hence the moderate P_C may suggest that extremely severe and chronic hypoxia is not an issue inside the gall. Both the CO_2 and activity P_Cs will serve as points of reference in subsequent discussions regarding oxygen levels inside the gall.

Chapter 3: Modelling the oxygen environment within a goldenrod gall

Introduction

The gall's oxygen level is determined by oxygen supply into the gall and consumption by living tissue inside. For oxygen supply, the physical dimension of the gall should be a factor, since the rate of diffusion is affected inversely by the distance over which diffusion occurs. This requires the assumption that diffusion instead of bulk flow is the main method of gas exchange. Temperature inside the gall rises above air temperature in direct sunlight (Layne, 1993), which may be evidence that convective cooling inside the gall is weak. Therefore, convective gas flow required for convective cooling should also be weak, and in this study only gas diffusion in the gall is considered.

Diffusion coefficients are also necessary for calculation of diffusion rates. Since the gall is divided into distinctive tissue layers, there is reason to model each layers with unique diffusion coefficients. In particular, diffusion may be slower through the dense outer skin than through the interior of plant organs (e.g. Abdul-Baki and Solomos, 1984; Lammertyn *et al.,* 2001). Changes also occur in other layers over development: After the gall reaches full size, cytoplasm content in the cortex layer becomes reduced, which may be adaptive through reducing the cost of gall production for the host (Weis *et al.*, 1989). As a result, the layer takes a harder but sponge-like texture (personal observation), and should be expected to show diffusion characteristics different from cytoplasm-rich tissue including young cortex itself. The cortex is the thickest tissue layer and takes up the most volume (Weis *et al.*, 1989), so changes in this layer may have more profound effects on oxygen level.

Oxygen consumption must also be taken into account as an estimate for the amount of diffusion at a steady state. The change in the cortex layer may affect oxygen consumption, since tissue with little cytoplasm should have reduced total metabolism. Another factor that affects oxygen consumption would be temperature due to temperature dependency of metabolism. Temperature in *E. solidaginis* galls largely follows air temperature, but increases further under direct sunlight (Layne, 1993). Temperature-dependent metabolism is also shown by the larva in past research (Layne and Eyck, 1996) and during larval respirometry in this study (Chapter 2). Lastly, the larva's demand for oxygen would also increase as it grows due to increasing body mass.

An important approach in this study was measuring oxygen level in wild galls directly with oxygen probes (Chapter 4). However, in preliminary trials very young galls were too small and fragile for our probes, so field sampling did not cover the entire course of gall development. Variability in field conditions, such as larva survival (impossible to assess without opening the gall and eliminating the natural oxygen condition), could also make relevant data difficult to obtain. Hence, besides field measurement, I also attempted to develop a simplified model for gas exchange inside the gall. From the model I intended to develop expectations regarding how the gall's oxygen level may change during the course of gall and larval development and due to outside conditions.

Method

Basic calculation

A simplified gall was modelled as a series of three concentric spherical shells, each with different gas permeability and respiration rate. First, the two shells on the outside represented the vascular layer and the mature, spongy cortex layer, respectively. For simplicity, the third shell represented all remaining tissue, including the fresh, cytoplasm-rich portion of the cortex layer, the parenchyma, and nutritive layers in which cytoplasm reduction does not occur until after plant death.

At a steady state, oxygen diffusion into a particular shell is assumed to equal to the total oxygen consumption by all living tissue inside the outer radius (r_o) of the shell, including subsequent shells and the inhabitant. Calculation for each shell follows Seymour and Bradford (1987)'s method (equation 3.7), which could be deduced from basic principles in heat conduction physics that have analogs in diffusion formulae. The basic equation is (equation 1.2, Kreith and Bohn 1997):

$$q_k = \frac{Ak}{L}(T_{hot} - T_{cold)} \quad (3.1)$$

Heat conduction rate (q_k) is proportional to the area (A) over which conduction occurs and to difference in temperature ($T_{hot} - T_{cold}$) or ΔT, and inversely proportional to distance of conduction (L). The difference in temperature (ΔT) can be substituted by a difference in gas pressure (ΔP), and the thermal conductivity (k) can be replaced with the diffusion coefficient (D), therefore arriving at the equivalent formula in diffusion physics:

$$\dot{V} = \frac{AD}{L}(P_{high} - P_{low}) \quad (3.2)$$

In heat conduction, $\frac{L}{Ak}$ is expressed as thermal resistance R_k, and its reciprocal, $\frac{Ak}{L}$, is thermal conductance K_k (equations 1.3 and1.4 in Kreith and Bohn 1997). Thermal resistance of a spherical shell (R_{th}) is calculated as: (equation 2.48 in Kreith and Bohn 1997):

$$R_{th} = \frac{r_o - r_i}{4\pi k r_o r_i} \quad (3.3)$$

Substitute R_{th} for $\frac{L}{Ak}$ in 3.3, and the reciprocal becomes:

$$\frac{Ak}{L} = \frac{4\pi r_o r_i k}{r_o - r_i} \quad (3.4)$$

The difference between outer and inner radii (r_o - r_i) is the shell's thickness, which is the distance over which heat conduction occurs (L). Substitute (r_o - r_i) with *L*, so that *k* and *L* from both sides of the equation cancel out, then 3.4 becomes:

$$A = 4\pi r_o r_i \quad (3.5)$$

Substitute 3.5 into 3.2, and express terms specifically for oxygen:

$$\dot{V}_{O_2} = \frac{4\pi r_o r_i D_{O_2}(P_{O_2(out)} - P_{O_2(in)})}{L} \quad (3.6)$$

$\dot{V}o_2$ is the diffusion rate for oxygen, P_{O_2} is the partial pressure of oxygen, and D_{O_2} is the diffusion coefficient of oxygen in shell material. Rearrange 3.6 to solve for oxygen pressure inside the shell:

$$P_{O_2(in)} = P_{O_2(out)} - \frac{\dot{V}_{O_2} L}{4\pi r_o r_i D_{O_2}} \quad (3.7)$$

Equation 3.7 was used once for each shell, three times in total for one simplified gall. For the skin layer, $P_{O_2(out)}$ was assumed to be 20.9% of atmospheric pressure. For the subsequent two shells, $P_{O_2(out)}$ equals to $P_{O_2(in)}$ of the previous shell. $P_{O_2(in)}$ of the innermost layer is referred to as the final result. Shell thickness data were based on galls used in direct oxygen measurements of Chapter 4. Rate of oxygen diffusion ($\dot{V}o_2$) and diffusion coefficient (D_{O_2}) was determined as follows:

Total rate of diffusion

In a steady state, $\dot{V}o_2$ should be equal to the total oxygen consumption by all tissue inside a particular shell, including subsequent shells and the inhabitant. Respiration rates for the larva and different gall tissue types were estimated separately:

Larval respiration rate data came from Chapter 2, calculated from rate of CO_2 production (assuming a respiratory quotient of 1) at the very beginning of the critical P_{O_2} experiments when oxygen level was near normoxia. Gall tissue respiration was measured in a plastic syringe modified for this purpose. Several mm were cut from the tip of the syringe, so that the Neofox FOXY-R oxygen probe fit tightly inside with a piece of soft

tubing around the probe to form an airtight seal. Vacuum grease was also applied near the probe tip before insertion to ensure sealing. Tissue sample would be placed inside the syringe on a piece of moist tissue paper to reduce sample dehydration. A small perforated plastic container with soda lime (Alfa Aesar®, 8-12 mesh, with indicating color) was also added to absorb carbon dioxide in order to prevent potential effects on respiration due to CO_2 accumulation. The entire assembly was placed inside a temperature-controlled chamber (the one used for larva respirometry in Chapter 2), and the change in oxygen level was recorded until the oxygen level fell below 10%. The rate of oxygen consumption per unit of mass ($\dot{M}O_2$) was calculated as:

$$\dot{M}_{O_2} = \frac{\Delta P_{O_2} \cdot V}{t \cdot m} \quad (3.8)$$

ΔP_{O_2} is the change in oxygen level (initial P_{O_2} - final P_{O_2}) inside the syringe during time t. Initial P_{O_2} was the atmospheric level of 20.9%, and final P_{O_2} was usually taken as 10%. *m* is raw mass of the plant tissue sample. V is the volume of gases inside the syringe, calculated as the volume of the syringe (set to between 1-2 mL) minus that of all objects within (soda lime container, moist paper, and the sample itself). Therefore, in the modelling of gall P_{O_2}, for each shell, oxygen consumption rate was calculated as mass-specific respiration rate of the tissue multiplied by mass, which was in turn calculated from average density of tissue samples taken to represent each shell.

Final respiration rates used in the calculation were corrected for temperature dependency. Most larval and plant respiration measurements (on all three modelled tissue types) took place at 25˚C. Some larval measurements and plant skin trials also took place

at 35˚C in order to estimate the Q_{10} value. Temperature-specific respiration rates were calculated from respiration rate at 25˚C and the Q_{10} value from the basic Q_{10} formula:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2 - T_1)} \quad (3.9)$$

R is respiration rate at temperature T. Rearrange to solve R_2, when R_1 and T_1 represent the situation at 25˚C:

$$R_2 = R_1 \cdot Q_{10}^{(T_2 - T_1)/10} \quad (3.10)$$

For this model, metabolic depression of plant and larva in hypoxia was not taken into account. Therefore, it is likely that oxygen consumption in hypoxia would be overestimated, and so would be the extent of the most severe hypoxia predicted. Negative values, which appeared in preliminary calculations, should be regarded as expectation for total anoxia (0 kPa) and would be expressed as such in subsequent figures.

Estimating tissue-specific diffusion coefficients

Diffusion coefficient was measured using custom equipment shown in Figure 3-1, largely following methods used by Seymour *et al.* (1986). The chamber (chamber height 12.7 mm, diameter 8.6 mm) was largely filled by a rubber plug (length 11.1 mm, diameter 7.5 mm) of known volume to minimize chamber size, in order to mimic the ideal situation (the anoxic chamber being analogous to the center of a piece of anoxic material) demanded by the formula. The oxygen probe passed through the center of the cylinder, and minimal space around the cylinder remains for nitrogen input. Before each trial, the sample was placed in 0.01 molar sodium azide solution for 30 minutes in order

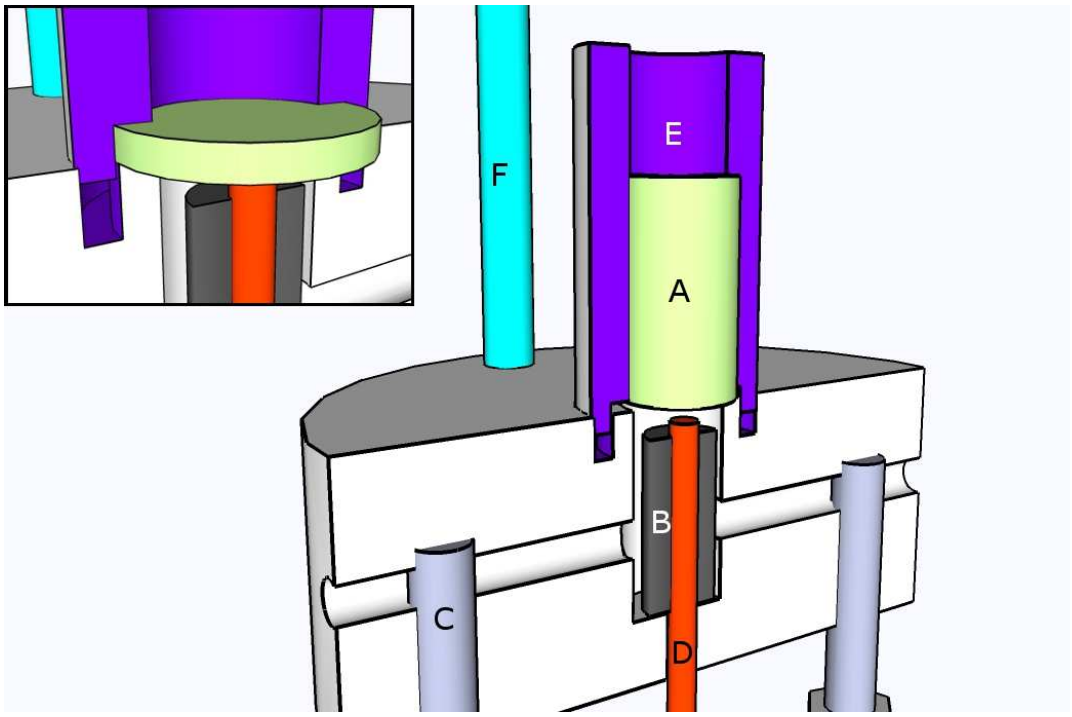

Figure 3-1: Equipment used to measure coefficients of diffusion in gall tissue. Only half of most parts were shown. Oxygen in lab air would diffuse through the tissue sample (A) into a chamber (B) initially filled with nitrogen via tunnels controlled by screw valves (C), in which the change of oxygen level was monitored with a Neofox FOXY-R oxygen probe (D). The chamber's volume was minimized by a rubber column in order to mimic the situation in Seymour *et al.* (1986) where little air space remains on the bottom of the sample column where the probe rests. Thin (less than 1mm) skin samples (small diagram) would be held between a metal tube (E) and the chamber body, while thicker samples of other tissue types (main diagram) would be held inside the tube with the side sealed with vacuum grease (purple surfaces on the diagram). The tube was held in place by nuts and a metal plate (not shown) on long bolts connected to the main body (F). Design modelled in Google Sketch 7 (not to scale).

to reduce metabolism. A 0.001 molar solution inhibited respiration of oat coleoptile within 1 hour (Kelly, 1947). In preliminary tests gall tissue sample changed in color and texture after prolonged exposure, so exposure to higher concentration for a shorter period was chosen. Thereafter the sample was allowed to dry to ± 0.01g of its original mass before being mounted in the core holder. The inner chamber would be flushed with nitrogen for 30 minutes. This step was the attempt to saturate the chamber and sample with nitrogen and set up the initial assumption for the calculation, but timing was limited in order to keep the sample fresh. Then, with gas valves closed, oxygen was allowed to diffuse through the tissue into the chamber. Diffusion coefficient was calculated from time taken for the oxygen level to rise to half of atmospheric value ($t_{1/2}$) according to the formula used by Seymour *et al.* (1986):

$$D_{O_2} = \frac{F_o \cdot L^2}{t_{1/2}} \quad (3.11)$$

F_o (the Fourier modulus) is dependent on the Biot number, which is an indication of diffusion into the material concerned at the interface. Here diffusion into the sample is assumed to be much faster than diffusion through the sample, and Biot number can be assumed as very large or infinity. Under this assumption, F_o for $t_{1/2}$ would be approximately 0.375 (Apprendix D, Figure D.1 in Incropera and Dewitt 1996), which is the value used by Seymour *et al.* (1986).

Unfortunately, during several runs the oxygen level never rose to over 10% during the time that could be given realistically to the experiment. In this situation, an alternative method was used to estimate diffusion coefficient. Oxygen level in the chamber stabilized at a low level, suggesting poor inhibition of sample metabolism. The calculation followed

a rearrangement of formula 3.2 solving for D, with A and L substituted by cross section area and thickness of the sample respectively:

$$D_{O_2} = \frac{\dot{V}_{o_2} \cdot L}{A\left(P_{O_2(out)} - P_{O_2(in)}\right)} \quad (3.12)$$

Following the development of a hypothetical gall

Oxygen level within the chamber of a hypothetical gall was calculated for 160 days, representing dates from late May to the end of October. For the first 100 days (May 25th to September 1st, representing time when the majority of larval development takes place), the severity of hypoxia was described with respect to the average CO_2 and activity P_Cs (9.5 and 3.6 kPa respectively) estimated in Chapter 2, as the number of days on which the calculated O_2 level falls below the P_Cs. Development-dependent parameters of the hypothetical gall were estimated from information gathered during field measurements (Chapter 4) as follows:

Air temperature: Similar to climate data used in Chapter 4, daily temperature data from the St. Catharines Airport weather station (WMO ID: 71262) were downloaded from the National Climate Data and Information Archive (http://www.climate.weatheroffice.gc.ca). Temperature data from the year 2010 were used for the hypothetical year. The maximum temperature for each day was used in calculation to represent the condition that leads to the highest metabolic rates.

Gall size: Once visible, the gall reaches its full size rapidly in less than a month, and after that the size barely changes. Not enough early growth data were collected during this study to fit a sigmoid curve, so a simplified curve between initial emergence

and final size was modelled by a quadratic curve ($y = \alpha x^2+bx+c$) with a negative term α, representing the slowing-down of host plant growth as the plant ages (Horner *et al.*, 1999). The intercept at the y axis (0, c) was the goldenrod stem thickness assumed to be a uniform 3 mm, and the curve's peak represented the maximum size at 25 days after gall formation. Subsequent gall sizes were all assumed to be at maximum. For a typical healthy gall, a final diameter of 25 mm was assumed.

Larval mass: Parameters for a generalized logistic curve were estimated from larva mass data collected from galls used for direct oxygen level measurements in the year 2010 (Chapter 2). Estimation was carried out using the *gcFitSpline* function in R package *grofit* (Kahm *et al.*, 2010). Then a series of values was calculated using the *logistic* function in the same package.

Chamber size: The chamber is simplified as a sphere. It expands through tissue consumption by the larva, so its volume is assumed to be proportional to larval mass (m), i.e: $m = \alpha \cdot \frac{4}{3}\pi r^3$. Constant α was solved by substituting in the maximum larval mass from the hypothetical growth curve and the maximum chamber radius observed during field measurements (2.5 mm). Then the equation was rearranged to solve for r for every hypothetical date: $r = \sqrt[3]{\frac{3m}{4\alpha\pi}}$.

Gall tissue development: Skin thickness for the hypothetical gall was represented by the average of all skin thickness data from 2011 field measurements (skin thickness was not collected in 2010). The development of cytoplasm-poor cortex was expressed as a ratio, hereafter called the dry ratio (DR) for convenience, calculated as the ratio of dry

cortex thickness (Figure 1-2, part B) to gall wall thickness excluding skin (Figure 1-2, part B + C). Based on field observations, DR was assumed to increase linearly from 0 to 0.6 between 18th of June and 3rd of July, and then continue to increase and reach 0.9 on 1st October, and remain constant thereafter. Dry and fresh thicknesses were then calculated from total pith thickness and DR on the corresponding date. Plant death was assumed to occur on 1st October, after which plant respiration was calculated as zero.

Importance of parameters in the model

The contribution of individual parameters to potential variation of the resultant P_{O_2} was assessed with one-way sensitivity analyses. Such analyses involve modifying one parameter at a time and looking at the resultant change in the final result. Two sets of parameter were used: The first set involved taking averages of applicable parameters (gall size, skin thickness, dry ratio, animal mass and temperature) over all galls used in field measurement (Chapter 4) to calculate an oxygen level that represent a "typical" gall. The second set was taken from the point in the hypothetical gall's development that showed the most severe hypoxia. Parameters in the model were divided into 3 types and modified accordingly:

I. Diffusion coefficient, respiration rate, and density of plant tissue, as well as animal respiration, used the mean estimate from previous experiments in this study as the baseline. Respiration rates at a particular temperature were affected by temperature and Q_{10}. For the analyses, the baseline values were modified by ± 20%.

II. Parameters expected to show seasonal and developmental variation, including gall radius, chamber radius, skin thickness, animal mass, DR and temperature, used the mean of all data from field measurements as the baseline. In addition to ± 20% modification as in part I, these parameters were also modified by ± standard deviation to represent the relative amount of variation possible throughout development.

III. Dry and fresh tissue thicknesses were expected to affect the calculated chamber oxygen level. However, their thicknesses change through the drying process in the cortex layer, and changing one would require changing the other or even other parameters such as gall size. Since changing only one of these parameters is difficult, separate sensitivity analyses were not carried out, and their effects were represented by DR.

Besides these analyses, parameters were modified in the complete development curve to calculate three additional curves to illustrate the removal of certain processes:

1. Cortex does not develop into the cytoplasm-poor state, expressed as DR = 0% throughout the hypothetical growth period.
2. Temperature is constant at 25 °C.
3. Gall possesses a large chamber that reaches maximum radius of 0.25 cm on the third day in simulation.

Results

Tissue characteristics

The results are summarized in Table 3-1. Dry cortex was the least dense and fresh tissue had the highest density. At 25 °C, fresh gall tissue showed significantly higher respiration rates than dry cortex tissue, while the vascular layer showed no significant difference from the other two layers. Fresh tissue also showed the greatest variability in respiration rates. Temperature dependency was observed for skin tissue with a Q_{10} of approximately 1.8 between 25 °C and 35 °C. As in Chapter 2, most galls were collected from Area B (Figure 1-3), but tissue from 7 galls from Area A collected in field measurement were also used. Three among them were used at a later date, and kept as cuttings with the bottom in water before the trial. No data from these 7 galls were considered outliers (beyond mean ± 2 standard deviations of Area B data), so subsequent calculations used data from all galls.

Measuring diffusion constant

A total of 21 trials were carried out, with 6 on the vascular layer (skin), 10 on "fresh" gall tissue and 5 on dry cortex tissue after autolysis. In 8 fresh trials and 4 skin trials, oxygen level did not reach half of atmospheric level in the time that could be allocated to the experiment (Figure 3-2). All 4 problematic skin trials and 6 problematic fresh trials had oxygen level increasing to over 1%, and the alternative calculation (equation 3.12) was used.Oxygen level in all dry tissue trials reached at least half of atmospheric level over the time allotted. Six trials used gall tissue from Area A instead of Area B, but most of them belonged to the problematic trials, and the small number of

Table 3-1: Tissue density and oxygen consumption data[a] used in modelling.

Tissue type	Temperature (°C)	Sample size	Density (g/cm^3)[b]	Oxygen consumption (mL/g/h)[c]
Fresh nutritive and cortex	25	9	0.916 ± 0.062	0.287 ± 0.122
Dry cortex	25	5	0.353 ± 0.034	0.181 ± 0.050
Skin	25	6	0.756 ± 0.106	0.273 ± 0.076
	35	3		0.490 ± 0.039

[a] Mean ± standard deviation

[b] Significantly different between all tissue types: one-way ANOVA $F = 82.1$, $p < 2.28 \times 10^{-10}$, Tukey's HSD all pairwise p values < 0.001

[c] Significantly different between fresh and dry tissue: Tukey's HSD $p = 0.045$

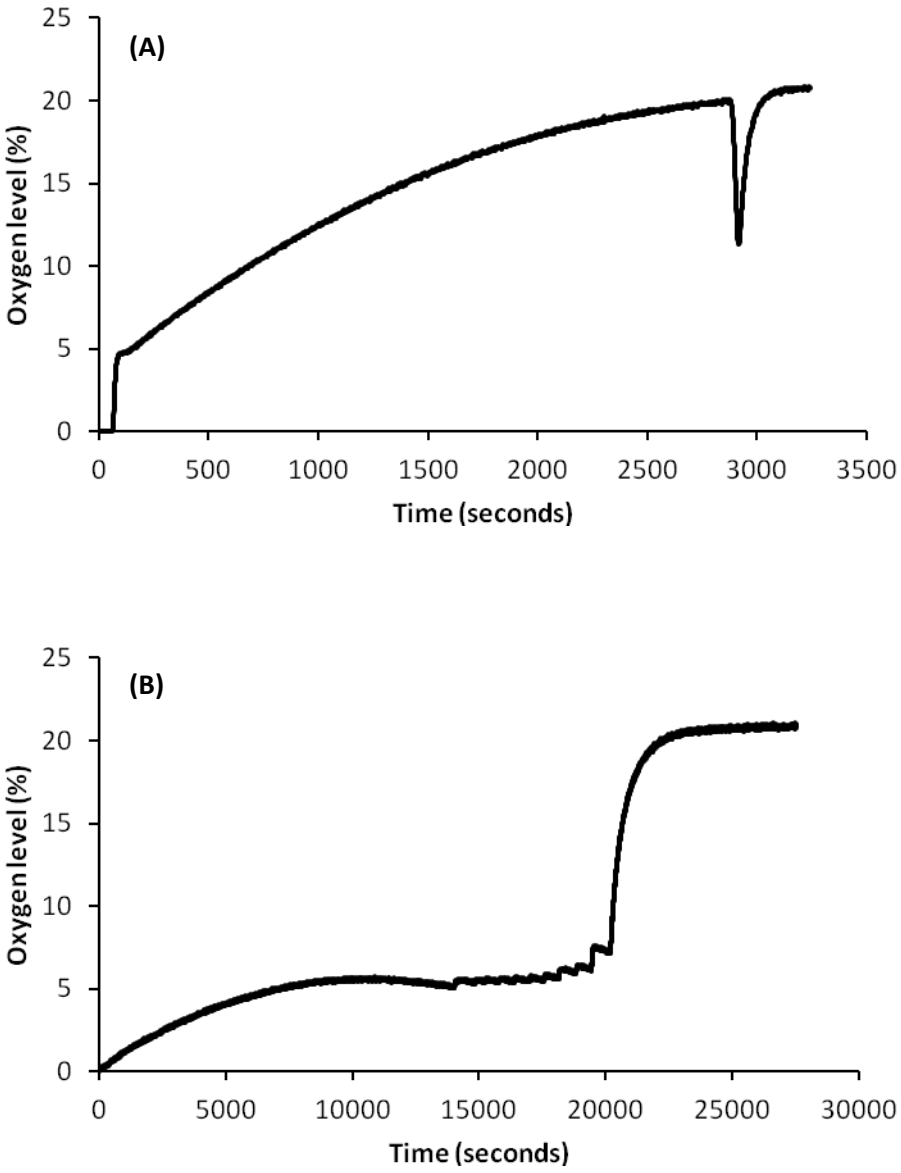

Figure 3-2: Sample plots of diffusion coefficient trials. A: A trial with dry cortex, with oxygen level eventually recovering to near atmospheric level. B: A trial with fresh gall tissue. Oxygen level in the chamber did not return to near atmospheric level until a long time later, likely due to leaks following sample drying (at time = 20000). The initial stable period may represent incomplete inhibition of sample tissue respiration, causing a steady state to develop between respiration and diffusion.

remaining trials also prevented meaningful comparisons. One of them was a dry tissue trial, and was not considered an outlier.

Within both methods, average D_{O_2} was lower in skin tissue than for fresh gall tissue. D in dry gall tissue was higher than for the other two tissue types determined with the standard method. Average values between methods differed greatly by 2-3 times, but the methods have different assumptions in the first place. Fresh tissue D values from the standard method were most similar to the D value of oxygen diffusion in water, and also to values determined for pear flesh by Lammertyn *et al.* (2001) (while other plant tissue D values in Table 3-2 could be much higher). Skin values were also close to pear skin D values from the same study, and were lower than flesh/fresh tissue values by approximately one order of magnitude. For dry cortex no previous data were found for comparable tissue. So although only a few samples were available for the standard calculation, they had to take precedence over the alternative method values in calculation.

However, fresh tissue D values calculated from half-time were available from too few samples, and the large variation of dry tissue D values was suspicious. As a backup, oxygen level over the development of a hypothetical gall (next section) was calculated with both gall tissue D values from the half-time based calculations (equation 3.11) and pear flesh D values from Lammertyn *et al.* (2001), which were numerically similar to the average half-time based gall tissue D values. Dry tissue D in this case was assumed to be three times of the fresh tissue D, similar to the amount of difference between fresh and dry gall tissue D values.

Table 3-2: Coefficients of diffusion determined in various plant tissues.

Species and cultivar	Tissue	Gas	Original value reported	Converted units to cm^2/s	Temperature (°C)
Eurosta solidaginis gall on *Solidago altissima*	Skin	O_2		2.61×10^{-6}	25
	Dry cortex			8.39×10^{-5}	
	Fresh cortex and nutritive			2.5×10^{-5}	
Potato, Russet Burbank[a]	flesh	CO_2	2.65×10^{-4} cm^2/s	2.65×10^{-4}	27
	skin		7.26×10^{-7} cm^2/s	7.26 × 10-7	
Apple, Braeburn[b]	flesh	O_2	0.0012 cm^2/s	0.0012	20
Apple, Cox's orange pippin[b]			0.0027 cm^2/s	0.0027	
Pear, Hosui[b]			0.0003 cm^2/s	0.0003	
Pear, Kosui[b]			0.0011 cm^2/s	0.0011	
Nectarine, Red Gold[b]			0.0017 cm^2/s	0.0017	
Nectarine, Sunglo[b]			0.0018 cm^2/s	0.0018	
Pear, Conference[c]	flesh		1.71×10^{-9} m^2/s	1.7×10^{-5}	
	skin		2.84×10^{-10} m^2/s	2.8×10^{-6}	
Quercus suber (cork oak)[d]	cork		1.1×10^{-9} m^2/s	1.1×10^{-5}	25

Sources: [a] Abdul-Baki and Solomos (1994), [b] Rajapaske *et al.* (1990), [c] Lammertyn *et al.* (2001), [d] Lequin *et al.* (2012).

Development of a hypothetical gall

Modelled oxygen level within the gall was near normoxia initially, but a drop occurred as the hypothetical gall increased in diameter (Figure 3-3). The most severe predicted hypoxia of about 1.2 kPa occurred on day 24, 1 day before maximum gall size was reached. Oxygen levels rose above 10 kPa on day 29 and 19 kPa on day 46, and continued to increase until the assumed plant death when small fluctuations in oxygen level disappeared. With regard to larval tolerance to hypoxia, oxygen levels fell below the average CO_2 P_C (9.5 kPa) for 13 days, and below the average activity P_C (3.6 kPa) on 4 days. These represent less than 1/10 of the total length of simulation (160 days). When D values for pear tissue by Lammertyn *et al.* (2001) were used, the number of days below P_Cs increased to 21 and 9 days respectively. Day-to-day fluctuations occurred before assumed plant death, due to respiration being influenced by temperature. Since temperature used in the calculation was the daily maximum, oxygen consumption would be higher than average due to temperature dependency of metabolism, which leads to more severe hypoxia. Gall temperature may still rise above air temperature in direct sunlight (Layne, 1993), but the calculated oxygen level would represent the most severe estimate of hypoxia in a given day based on known temperature data.

Sensitivity to parameters

Results of the analyses are summarized in Figure 3-4. Oxygen level in the "typical" gall calculated from average parameters was 19.1 kPa. When percentage modifications were made, respiration, diffusion and density of the skin and the dry tissue layers only

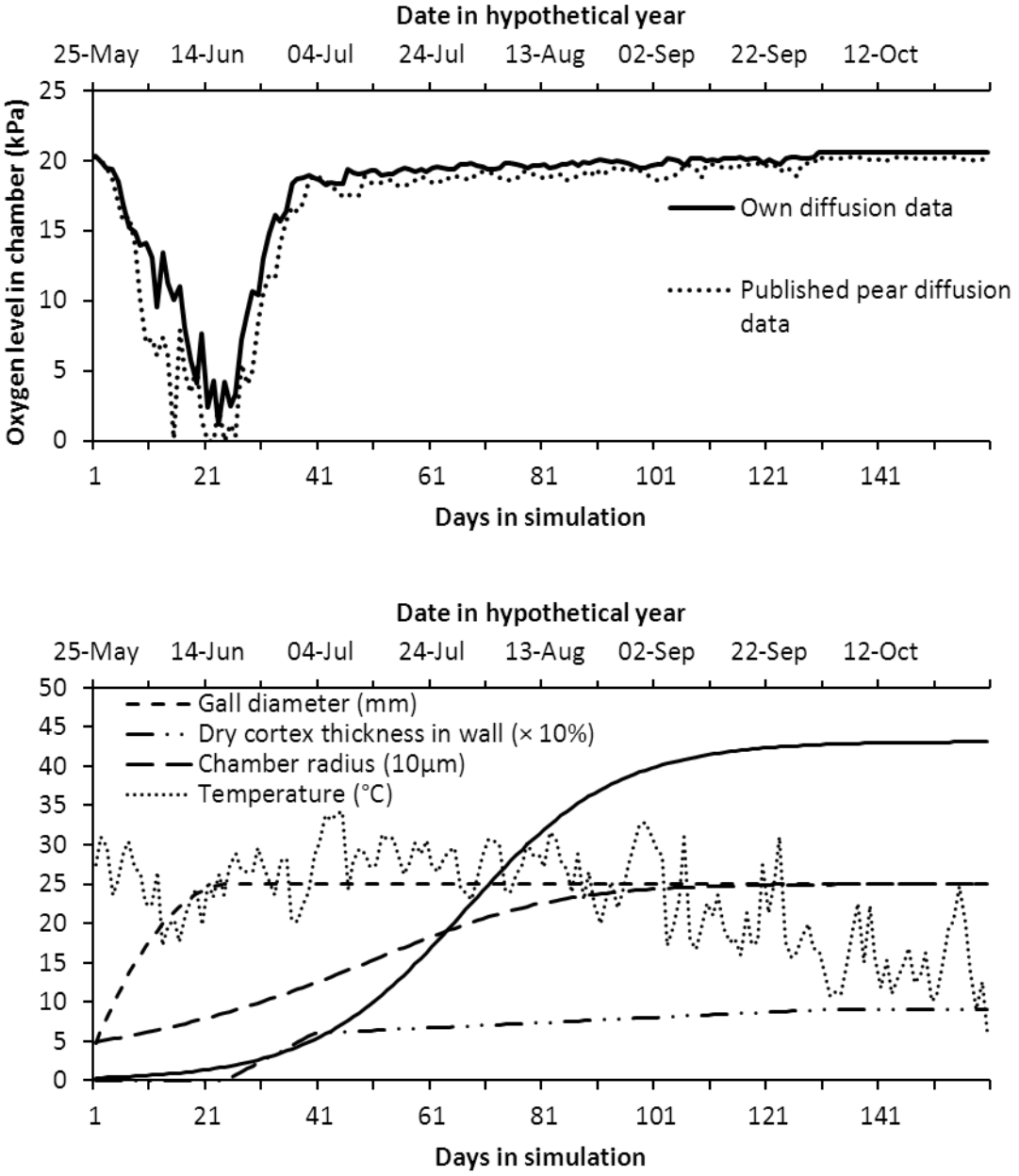

Figure 3-3: Oxygen level in a hypothetical simplified gall. Calculated over the summer and autumn growth season. Alternative calculation used pear tissue diffusion constants published by Lammertyn *et al.* (2001). Time- and development-dependent parameters are plotted for comparison.

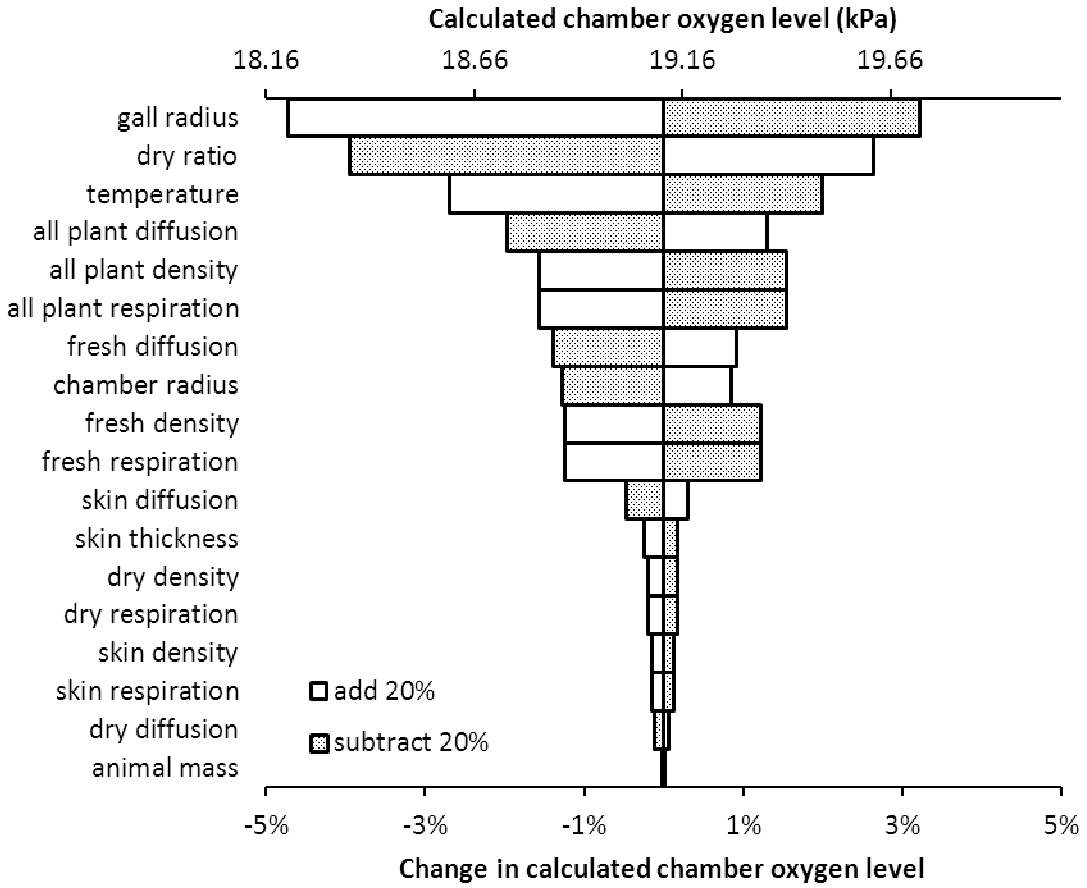

Figure 3-4 (A): One-way sensitivity analysis on a simplified model of gall oxygen level. The baseline was established to represent a "typical" gall with average parameters. The parameters were modified by adding or subtracting 20%.

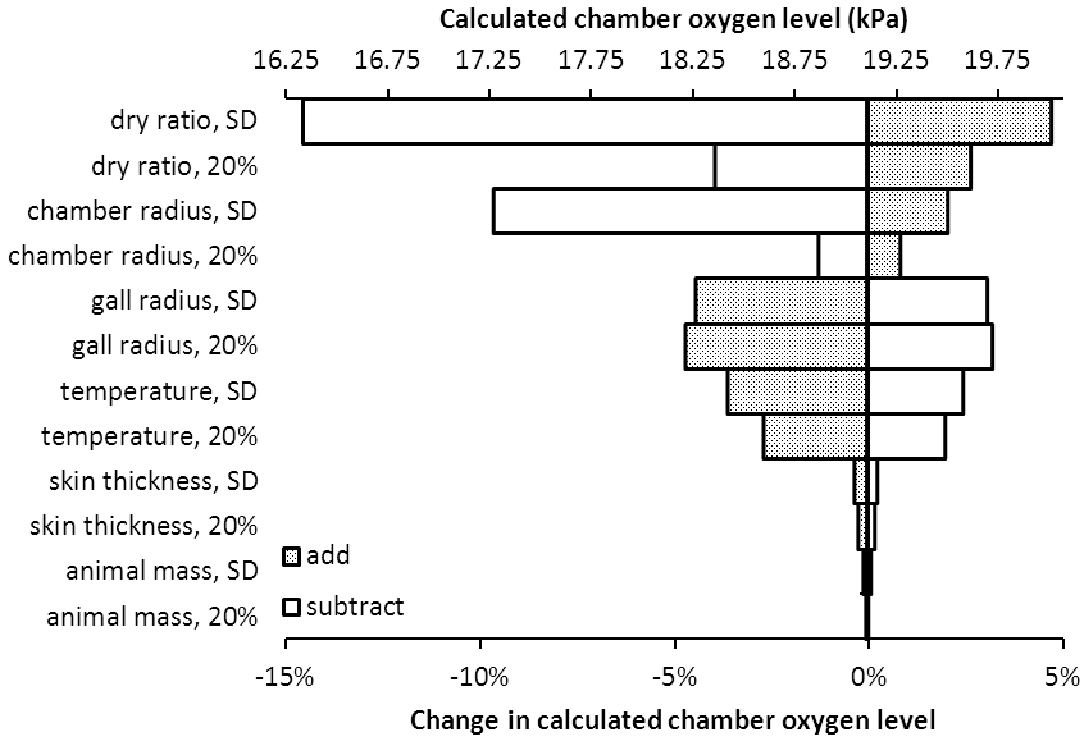

Figure 3-4 (B): For applicable parameters (those with data regarding change throughout gall development or natural variation), the analysis is repeated by adding or subtracting one standard deviation. Results are plotted against the 20% modification results to illustrate discrepancies in relative importance.

affected the oxygen level by less than 0.5%, unlike the same properties of the fresh tissue layer which showed effects of more than 1%. Change in animal mass was the least influential among all parameters. Changes in gall size, DR and temperature were the most influential (between 2.5% and 5%), even more so than fresh tissue properties.

For each parameter, variation over the entire growth season or developmental period was not necessarily the same, as assumed in the arbitrarily chosen 20% modification. Therefore, the importance of parameter influences assessed by percentage modification and standard deviation modification was different. Chamber radius and DR became the most influential parameters, while the importance of gall radius and temperature fell below the two parameters above. Modification of DR led to the greatest change in predicted oxygen level, from 19.1 kPa to 16.3 or 20 kPa. Magnitude of effects from gall radius and temperature did not change as significantly as in chamber radius and DR, but such comparison is unlikely to be meaningful since modification of 20% was chosen arbitrarily. Skin layer thickness and animal mass remained least influential on developmental timescale. For both SD and percentage modifications, magnitude of oxygen level reduction was greater than magnitude of increase, likely due to the results being limited by the atmospheric oxygen level.

Parameter modification on the most severe hypothetical hypoxia (1.2 kPa) led to greater changes in oxygen level relative to the baseline, so the results (Figure 3-5) are presented as oxygen level (kPa) rather than percentages (which would lead to figures like 900%). Simulated cortex drying did not start at the time, so parameters regarding dry tissue were excluded from the percentage analysis (parameter = 0 before adding or subtracting). The relative importance of parameters largely stayed the same. Except for

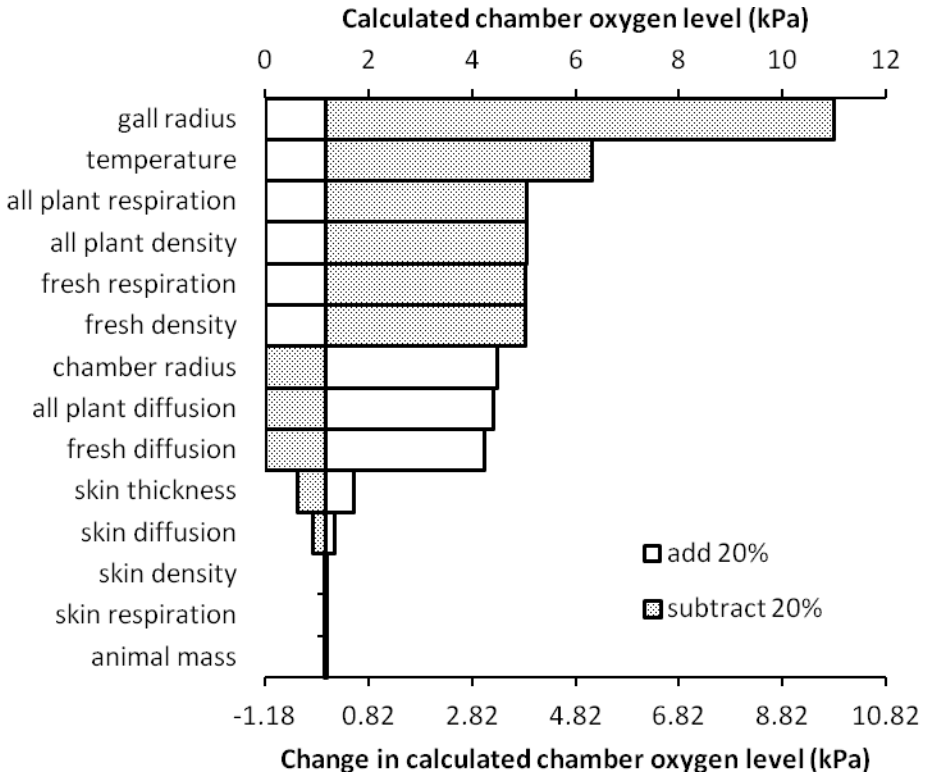

Figure 3-5 (A): Sensitivity analysis applied to the most severe hypoxia modelled. Under severe hypoxia, changes in parameter caused greater change in predicted oxygen level. Dry tissue parameters were excluded because cortex drying has yet to start.

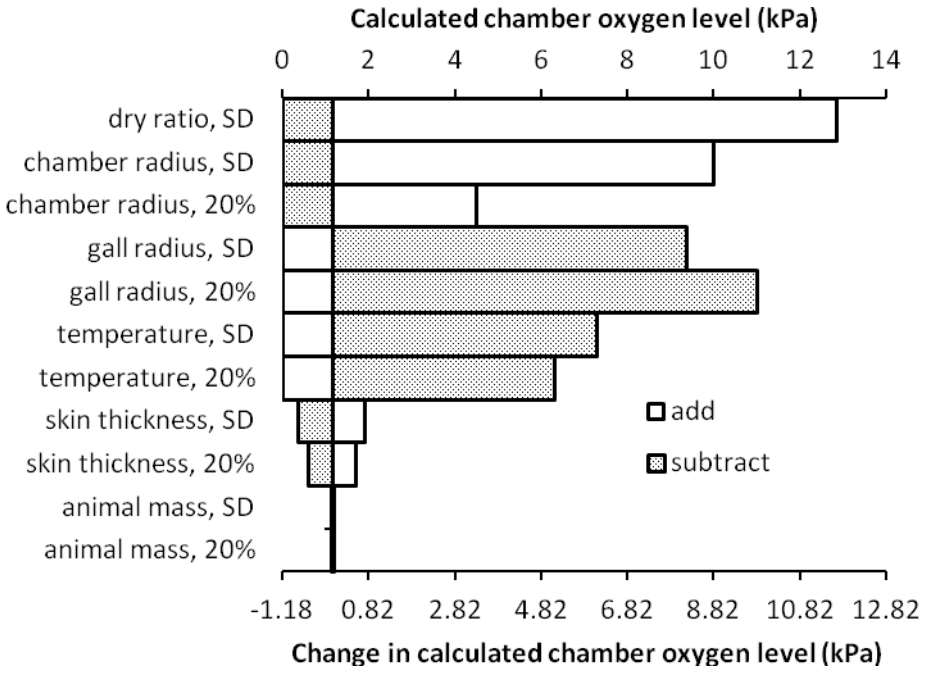

Figure 3-5 (B): SD modifications for the most severe hypoxia during development of the hypothetical gall. The difference in relative importance from percentage modification was similar to the "typical" gall, but the magnitude of change in predicted oxygen level was again larger.

the least influential skin and animal mass parameters, modifying other parameters by SD or 20% could increase gall oxygen level from the baseline of 1.2 kPa to between 4 and 13 kPa. Modification in the other direction also brought the oxygen level down to anoxia.

Keeping the cortex layer in the initial fresh state (Figure 3-6) led to more severe and lasting initial hypoxia that alleviated with plant death. The number of days with oxygen levels below the CO_2 and activity P_Cs increased to 38 and 13 days respectively, which is still less than 1/4 of the simulated growth season. A large chamber made the initial hypoxia less severe, and oxygen levels never fell below the higher CO_2 P_C. The constant temperature scenario removed abrupt fluctuations from the curve.

Discussion

Expectations and influences of gall hypoxia

Results from the simulated gall development suggested that hypoxia should only be limiting for the larva's aerobic respiration for a small portion of the growth season. The sensitivity analysis showed greater change in oxygen level for the same amount of parameter change (by one standard deviation) in the most hypoxic scenario than in the mildly hypoxic "typical" gall, which may suggest that severe hypoxia in the gall is less stable and more vulnerable to changes in parameters that affect gall oxygen level. Suggestion that hypoxia is not a problem for most of the time is consistent with findings from the previous chapter: P_C of *E. solidaginis* larvae was not particularly low among insects, and the animal likely was not adapted for living under severe chronic hypoxia. Dry cortex is less dense and less metabolically active than fresh gall tissue, so cytoplasm reduction in the cortex layer should indeed reduce oxygen consumption and permit

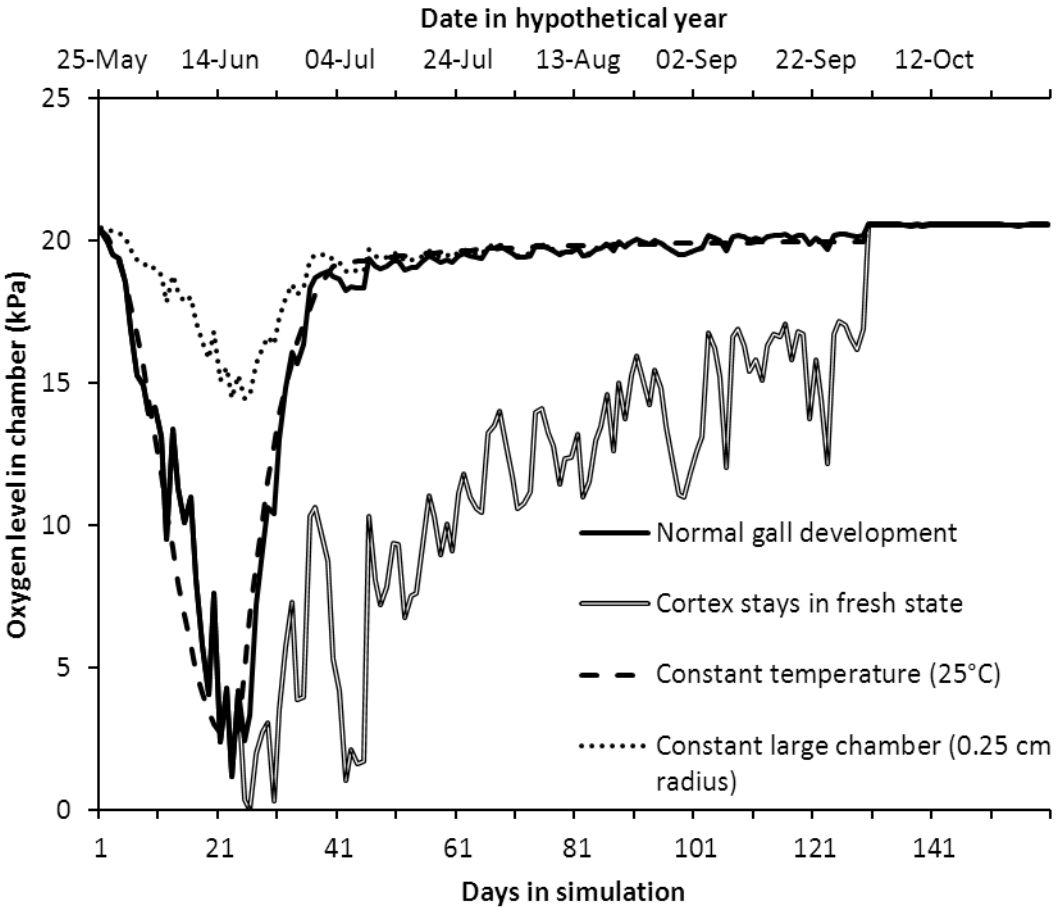

Figure 3-6: Modified models excluding certain developmental processes. Making the cortex remain in the initial cytoplasm-rich state led to more severe hypoxia, while a larger chamber made the hypoxia less severe. Modelling with a constant temperature removed day-to-day fluctuations in the curve.

greater oxygen diffusion, which makes diffusion more than sufficient to support aerobic respiration by gall tissue and the larva. When the drying process was removed, more severe hypoxia was seen throughout the hypothetical gall's development until plant death (Figure 3-6). Two of the most important parameters in the sensitivity analysis, gall size and DR, both involved the thickness of the fresh tissue layer. The skin layer however was less important, despite having metabolic rate and density comparable to the fresh layer in addition to low diffusion coefficient. Its large surface area and thinness may have limited its influence on oxygen exchange.

The insignificance of larval mass in the sensitivity analyses suggested that larval growth itself is unlikely to affect oxygen availability in the gall. When galls used in field measurement were fitted to the model (Chapter 4), expected oxygen consumption by the *E. solidaginis* larva represented on average only 0.3% of total consumption by all biomass in the gall, with a maximum of only 2.6%. Since *E. solidaginis* itself is the largest of the common gall inhabitants, respiration by other inhabitants is even less unlikely to have an effect without vastly different metabolic rates. This does not exclude the possibility that larva presence may affect gall physiology through physical or chemical stimuli.

Chamber size was important in the calculation likely because it represented the inner radius of the fresh tissue layer, and both the inner and the outer radii determined the effective surface area of the shell. Larger surface area in a large chamber means greater intersection between the intercellular spaces and chamber air, so that more diffusion could take place. However, it is unknown whether feeding could damage the interface, so

whether chamber affects gas exchange simply in terms of surface area needs to be studied further.

One other parameter considered in the planning stage was oxygen production from photosynthesis in gall tissue. Fresh tissue appears green, so it likely contains chlorophyll and is capable of photosynthesis. However, gall tissue is much thicker than the average leaf, so light is not expected to penetrate deep into gall tissue, and oxygen production at the surface may diffuse into the atmosphere more easily than inwards towards the larva. Haiden *et al.* (2012), studying the potential adaptive value of photosynthesis in gall tissue, found little contribution from photosynthesis to oxygen level in gall tissue.

Possibility of adaptation through the most influential parameters

The sensitivity analysis supported three most important parameters influencing gall oxygen level: total gall size, larval chamber size, and development of the cytoplasm-poor cortex layer. Could any of these be a basis for the larva's potential adaptation towards less severe hypoxia? Although total gall size could influence oxygen level greatly, it is under selection from predation, so reducing gall size to alleviate hypoxia may not be an adaptive option. There is likely no mechanism to produce chamber size variation, due to how the chamber is enlarged by feeding action, and therefore is linked to the larva's development. That leaves cortex development as a potential candidate. With the intention of looking for possible adaptation to tissue hypoxia via gall formation process, it is easy to think of this process as one such adaptation induced by the larva. However, information from plant and gall development may suggest otherwise. Cytoplasm reduction occurred in galls that suffered early larval death or parasitoid attacks, so it is

not entirely dependent on continuous stimulus from the larva. Cortex tissue is derived from stem pith tissue (Weis *et al.,* 1989) which also turns into a similar dry and spongy state in mature host plants (personal observation). This may be related to the process of pith autolysis, which is seen in many herbaceous plants, including *Solidago semperivens* [*sic*] (likely *S. sempervirens*, the seaside goldenrod) (Carr *et al.,* 1995). Pith autolysis is a similar process to lysigenous aerenchyma formation (Farage-Barhom *et al.,* 2008), which occurs in plant tissue under stress (including stress due to hypoxia) to produce extensive empty spaces that facilitate gas exchange (Evans, 2004). If pith autolysis in the healthy plant is carried over to the gall cortex, it is not surprising that the cortex would become more permeable to oxygen after the process. This may even suggest the lack of prolonged hypoxia is the default state in host stem tissue, including the gall which develops from the stem, and selection for larval adaptation to host tissue hypoxia may never have occurred. Like aerenchyma formation, the regulation of pith autolysis involves ethylene (Carr and Jaffe, 1995) which is released by plant tissue under stress, including stress from hypoxia (Evans, 2004). The model in this chapter expects more severe hypoxia in a developing gall, with gall enlargement leading to longer diffusion distance and fresh, growing tissue consuming more oxygen. Hence, a growing gall should naturally facilitate processes similar to aerenchyma formation (and pith autolysis too) without additional adaptation from the larva. Since the larva's feeding action leads to wounding response that increases oxygen uptake by the plant tissue (Raman, 2007), it may even be suggested that the larva's challenge is not to induce pith autolysis, but to maintain the cytoplasm-rich nutritive layer against pith autolysis (or at least the similar process in the gall) promoted

by the initial severe hypoxia in the gall and localized hypoxia in the nutritive layer due to wounding response.

Is there pressure for adaptation?

While the gall increases in size, *E. solidaginis* larvae grow slowly, increasing in dimensions from approximately 0.5 by 1.0 mm to 1.0 by 1.5 mm (Uhler, 1951). The most convenient explanation in this study's context would be metabolic depression caused by hypoxia. However, younger insects could also grow slowly due to slow feeding at small size. Also, Uhler (1951) documented the first ecdysis into the second instar at 0.7 by 1.3 mm on average, suggesting the first ecdysis takes place while the gall enlarges. Hence, the larva is apparently able to feed and develop during the initial hypoxia. So without further study, it is impossible to suggest young larvae are necessarily growing slowly due to metabolic depression. Conversely, it may be the case that alleviating hypoxia allows higher growth rate in older larvae. However, as explained above, cortex may exhibit cytoplasm reduction without larval intervention, including after larval death. Therefore, it is impossible to suggest whether the larva evolved to modify its gall in response to hypoxia during development.

Other adaptive values of cortex development

While the pith-autolysis-like process in the cortex layer might not be directly controlled by the larva, it is still a characteristic of the gall, the insect's extended phenotype. Perhaps it could be more specifically considered as a product of gallmaker-induced tissue growth and the pith autolysis process present in healthy host plants. On the evolutionary timescale, this process may have reduced the potential for prolonged and

severe hypoxia in bulky plant tissue, enabling the larva to evolve a gall size that provides the best protection against predators. Cytoplasm reduction has also been suggested as being adaptive through reducing the host's metabolic cost, therefore reducing the selection for defensive response against the larva (Weis *et al.*, 1989). This suggestion would not conflict with the suggestion regarding oxygen exchange, since reduction of metabolism and oxygen consumption are related. It would also be subject to the same arguments regarding evolution of the gall phenotype. Perhaps it would be useful to investigate the cytoplasm reduction process in more depth, to see whether it indeed is an extension of pith autolysis or is affected by the larva.

Improving parameter estimates in the model

Diffusion coefficient (D) experiments in living tissue encountered the most problems, mainly due to not being able to fully inhibit sample respiration. Admittedly the results would be inaccurate, but at least values from the standard method were similar to values from other plant tissues. In addition, underestimation of D values due to incomplete inhibition of plant sample metabolism only means that the calculated oxygen level would also be underestimations, so the actual hypoxia would be less severe than calculated. This trend would therefore not conflict with the lack of continuous severe hypoxia throughout gall development. Lastly, all problematic trials used skin or fresh gall tissue. Assuming the problem was indeed due to high sample respiration, the expectation that dry tissue would show lower respiration was supported.

Despite being a large gall, the goldenrod ball gall is still a much smaller structure (maximum diameter about 30 mm) than some other plant organs in which diffusion have

been measured (Table 3-2). Galls in this study grew in an uncontrolled environment, unlike the referenced plants which are agricultural products likely grown in controlled conditions. So it is possible that samples taken for a particular tissue did not all represent the same structure or the same stage and condition of development, and therefore contributed to the variability.

A second source of doubt was the plant tissue respiration estimates. In this study, tissue samples were cut out from living galls. As in the diffusion estimates, tissue separation could have been inaccurate. The sample would also invariably spend a significant period of time after being cut before results could be obtained, and would be kept outside the natural environment despite being under controlled temperature and high humidity. Therefore, it is unknown whether the sample's respiration truly represented respiration rates of intact galls' tissue.

On the other hand, observations regarding gall size and cortex development (collected as part of Chapter 4) should be quite reliable. So even if diffusion and respiration values are inaccurate, the general trend of the hypothetical gall's development (oxygen level showing an initial drop and then recovers to a relatively high level) would remain the same, and only calculated oxygen levels would be affected.

Chapter summary

The simplified model suggested that the greatest risk of hypoxia is during the gall's enlargement phase. Plant respiration should be unable to contribute significantly to gall hypoxia after extensive cortex autolysis occurs, and insect respiration is even less significant compared to plant respiration. The weakest part of all data used in the

calculation was the diffusion coefficients, due to inability to use best documented calculations or satisfy the assumption of the calculation actually used. However, values obtained were comparable to diffusion coefficients measured in other plant tissue, so the general order of magnitude for the comparison should be relatively accurate. Cytoplasm reduction in the cortex layer is expected to contribute significantly to alleviating potential hypoxia imposed by the bulky gall, but it would be rash to suggest this as an example of larval adaptation via extended phenotype, and the process may simply be an extension of the normal development process of the host plant stem.

Chapter 4: Direct measurement of gall oxygen level

Introduction

Plants lack specialized systems that transport respiratory gases. Oxygen level falls below atmospheric level in a variety of plant structures and could reach near anoxia (reviewed by Geigenberger, 2003). Hypoxic environments, such as waterlogged soil surrounding plant roots, affect tissue oxygen level (Armstrong *et al.*, 1994). Plant characteristics that lead to hypoxia include high tissue density, large size (bulkiness) and high metabolic rate such as during active growth (Geigenberger, 2003). More than one characteristic may affect a particular structure, such as in developing seeds with dense seed coat and high metabolism (Rolletschek *et al.*, 2001). Plants adaptations to hypoxia include regulated depression of metabolism and growth (Zabalza *et al.*, 2009) and developing tissue that facilitate gas exchange known as aerenchyma (Drew *et al.*, 2000).

Insects in living animal tissue are subject to hypoxia (Hoback and Stanley, 2001). When plant tissue exhibits hypoxia, insects burrowing within should experience hypoxia as well. Plants usually have lower respiration rates per unit volume than animals (van Dongen *et al.*, 2003), so responses that satisfy the plants' own respiratory needs do not necessarily match the animals' oxygen requirement. It is unknown whether this could be adaptive for the plant as a defensive mechanism, but humans utilize modified atmosphere with low oxygen content against insects in storage of agricultural products (Donahaye, 1990). Tissue growth in galls leads to increases in both diffusion distance and oxygen requirement (by more tissue and high metabolism during growth). Therefore, galls should

be at least as likely to develop hypoxia as healthy gall-bearing tissue. However, potential for hypoxia should still depend on the host plant tissue and gall characteristics.

The main question of this study, whether hypoxia in *Eurosta solidaginis* galls affects the larva, depends on the occurrence of gall hypoxia in nature. In Chapter 3, a mathematical model was established to make predictions about oxygen levels in the gall. The model is certainly unable to fully address variations in field conditions, so the collection of field data was an important part of the study. In this chapter, oxygen level inside wild galls was investigated by direct measurement with optical oxygen probes, and compared to expectations established in Chapter 3 by simplified mathematical modelling.

Method

Sampling took place in Area A (Figure 1-3), a patch of open field along a route leading to the campus' field maintenance facilities. Only apparently healthy galls without severe surface damage were chosen for direct measurement. Sampling may have been biased towards larger galls for the same reasons suggested for preferential attack by birds – larger galls are easier to find and are more likely to contain living *E. solidaginis* larva which makes the data more relevant to the study. Galls taken on the same day were at least 3 meters away from each other in order to reduce the possibility of sampling from the same plant genotype repeatedly.

Oxygen levels were measured using a Neofox FOXY-AF-series oxygen probe into the gall. This type of probe consumes no oxygen, and therefore should be more accurate in small spaces like the gall's interior. It was inconvenient to calibrate the probe in the field with nitrogen gas due to difficulty in transport. For unknown reasons, the probes

also did not respond well to the traditional method of calibration using anoxic sodium sulphite solution. After calibration in pure nitrogen, readings taken in the solution tended to be in the range of 5-10%. It was possible that the temperature of freshly-made solutions caused a discrepancy with the system's temperature probe. If this was the case, slow temperature change of a water solution (with high specific heat capacity) in the field could have delayed field operations. Therefore, instead of calibrating in the solution, the probe was put in pure nitrogen gas under a range of controlled temperatures in the lab, and raw probe readings in anoxia were recorded. When in the field, the raw reading corresponding to air temperature at the time was provided to the software. This, alongside the probe's reading in air, provided the data for a two-point calibration (requiring raw readings at normoxia and anoxia).

The probe was inserted through a tunnel in the gall wall cut out using a size 16G1 hypodermic needle aimed at the gall's center. In the 2010 season, an AL300 probe (300 μm in diameter) was used. A thin piece of tubing was placed around the probe for protection and to ensure a gas space remained at the tip of the probe, mimicking the gas space in which the larva resides. The probe itself was inserted through another needle that formed a tight seal with the tunnel. The gap between the rear of the needle and the probe was filled with Dow Corning vacuum grease. Before insertion of the needle containing the probe (change of needle was necessary due to blockage of the cutting needle by plant tissue), grease was also applied outside the needle near the tip for additional seal between the needle and the tunnel. After insertion, the probe was kept inside the gall for at least 15 minutes to compensate for the inflow of outside air into the tunnel. Readings were taken 10 times per second and averaged to produce one output per second.

Out of concerns about potential damage on the long and thin AL300 under repeated exposure to field conditions, sampling in 2011 used a larger FOXY-R probe (1000 μm in diameter) instead. The protective tubing outside the probe was abandoned, and grease was applied directly outside the probe near but not touching the tip. Gas space at the tip was made by slightly withdrawing the probe after insertion. The thick FOXY-R probe, being one solid piece, should reduce leak better than the three-part construction containing the smaller AL300 probe, although neither methods showed observable leaks in preliminary lab trials. The larger probe's thickness also forms a better seal with the tunnel. In addition, the small probe could be more vulnerable to environmental noise. Despite the dimensions of the FOXY-R, it could still not be used to penetrate gall tissue directly, so the possibility of gas flow into the tunnel between making the tunnel and inserting the probe remained. Hence the probe still remained inside the gall for 15 minutes after insertion.

Consistency between the two probes' readings was also tested, with emphasis on the effect of probe contact with tunnel (due to different probe diameter) and the effect of living plant tissue. Both probes were used to measure the oxygen level in a metal chamber (Figure 4-1). The chamber was filled with nitrogen and room air mixed at equal flowrates to yield a final oxygen concentration of approximately 10.45%. After turning off the gas flow and sealing the chamber, measurement took place through either a tunnel cut through a piece of fresh carrot in the core holder (to mimic a field trial with living plant tissue under controlled conditions) or a pre-made tunnel through the steel chamber

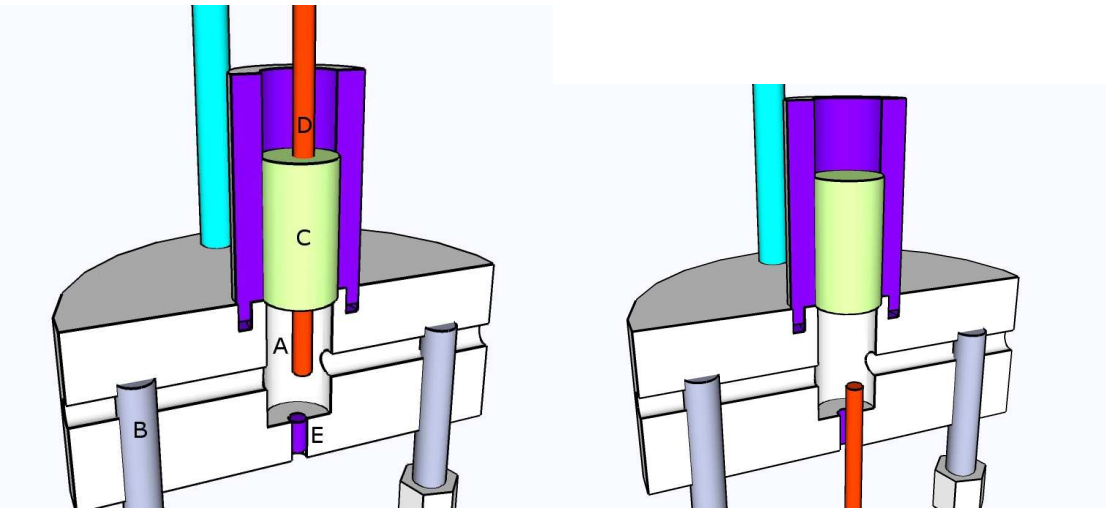

Figure 4-1: Equipment used to test reading consistency between probes. The equipment was originally designed for tissue diffusion coefficient measurements (Figure 3-1). Before probe insertion, the chamber (A) was filled with a gas mixture of known oxygen level, and then sealed off via screw valves (B). The gas mixture was nitrogen and room air mixed at an equal flowrate, with expected oxygen level of 10.45%. The plant sample is represented by a piece of carrot (C), through which both probes (D) were inserted (left) to mimic the field procedure, while the probe tunnel (E) was sealed by vacuum grease. Probes were also inserted through the chamber's probe tunnel (right) to control for potential effects from the probe's contact with plant tissue.

Table 4-1: Results for reading consistency tests between oxygen probes. No significant difference[a] from expectation was detected, and no difference due to probe change or tunnel material was found. Results from the carrot tunnel showed greater variation.

A. Two-tailed t-tests[b] for probe accuracy in a gas with known O_2 concentration.

Probe	Tunnel	Mean reading (%)	SD	t	p[a]	n
AL-300	Carrot	10.93	1.95	0.78	0.46	10
AL-300	Steel	10.05	0.59	2.13	0.06 n.s.	10
FOXY-R	Carrot	10.06	2.05	0.60	0.56	10
FOXY-R	Steel	10.45	1.02	0.00	1.00	10

B. ANOVA[c] on whether different probe or tunnel materials affected readings.

Source of variation	df	Sum of squares	F	p[a]
(Intercept)	1	1012.04	431.0867	$<2\times10^{-16}$ **
Probe	1	3.78	1.61	0.212
Tunnel	1	0.76	0.32	0.573
Interaction	1	4.03	1.71	0.198
Residuals	36	84.51		

[a] p value significance: n.s. $p < 0.1$, * $p < 0.05$, ** $p < 0.01$

[b] H_0: mean reading in gas mixture = 10.45%

[c] Two-way ANOVA with type III SS, calculated in R 2.12.0 using Anova() function from the *car* package.

wall, initially filled with vacuum grease (as a control for potential effect from the plant's injury response). Tunneling through carrot or the pre-made tunnel followed the procedure used for field measurements, and the probe tip stayed inside the tunnel. Results (Table 4-1) suggested that both probes gave accurate readings when surrounded by plant tissue, and the probe change should not interfere with comparison of results between years.

The following variables were collected along with field oxygen measurements on galls:

Environment

Temperature and air pressure were automatically recorded by the Neofox Viewer software. The prevalent weather during data collection (sunny or cloudy) was recorded manually. Recent precipitation was recorded as the number of days in which precipitation occurred within seven days before the sampling day, and within the sampling day itself (only the hours before sampling were taken into account). Hourly weather descriptions were retrieved from the St. Catharines Airport weather station (WMO ID: 71262) via the National Climate Data and Information Archive (http://www.climate.weatheroffice.gc.ca, abbreviated as NCDIA in subsequent discussion). The amount of precipitation was unavailable. The airport itself is nearly 10 km away from the university where samples were taken, and the two locations were on different sides of the Niagara Escarpment. To account for potential differences in local weather, a particular day would be recorded as raining if rain was observed personally at Brock University, regardless of the online data.

Host plant

Height of the goldenrod ramet and height of the gall on the stem were measured to the nearest cm. On the gall itself, the outside diameter at the "equator" and the diameter of the inner chamber (perpendicular to the larva's body) were measured with digital callipers. The total thickness of the gall's mature cortex (white and spongy part) and the inner fresh layer were recorded as a reference for the gall's development after reaching full size. In 2010, gall dimension readings were only recorded to the nearest 1 mm. In 2011, due to the additional goal of modelling gas diffusion across the gall wall (Chapter 3), measurements were made to the nearest 0.1 mm, and the thickness of the gall's vascular layer (skin) was also recorded.

Gall inhabitant

After opening the gall, animals present in the central chamber were identified and weighed to the nearest 0.1 mg.

Statistics to identify factors affecting oxygen level

Raw oxygen level data in fraction was multiplied by atmospheric pressure readings and therefore converted to kPa. The converted oxygen data were then analyzed with a linear regression with various parameters collected with the oxygen readings. Oxygen level data did not show a normal distribution, and transformation by $\log_{10}(x+1)$ did not bring them to normality (Shapiro-Wilk normality test, $p < 1\times 10^{-10}$). So oxygen level was analyzed without transformation, and the analysis was not intended to be used predictively. The most important parameters included in the initial model were then selected by Akaike Information Criterion (AIC) calculations. The initial model included the following parameters, without interaction terms:

year: 2010 or 2011, coded as 0/1 (whether the year is 2011).

date: days after first gall was observed, coded as integers.

phigh: plant height in cm.

diam: gall diameter in mm.

idiam: central chamber diameter, log-transformed

dryratio: percentage of gall wall thickness (excluding skin and central chamber) represented by dry cortex, with arcsine transformation

amass: mass of animal inside gall, weighed to the nearest 0.1 mg

penetrate: whether probe has penetrated the central chamber, coded as 0 or 1

pressure: atmospheric pressure (kPa) recorded by the Neofox system.

temperature: air temperature (°C) recorded by the Neofox system.

ppt: number of days in which precipitation occurred in the previous week. Precipitation on the sampling day before sampling time also counted as a day.

ghigh: height of gall on the goldenrod ramet, in cm.

afly: whether the gall inhabitant is *E. solidaginis*, coded as 0 or 1.

Comparison of direct results with modeling expectations

Besides results from the direct measurement, another oxygen level was also calculated for galls taken from the field following the model created in Chapter 3.

Parameters used directly in the calculation were: gall dimension and tissue thicknesses, air temperature, atmospheric pressure, and larval mass (only *E. solidaginis* larvae were considered). Vascular layer thickness was not recorded in 2010. Therefore for galls taken in 2010, the mean of all vascular thickness data from 2011 (0.77 mm) was used.

Result

Sample period and size

In 2010, the first galls were observed on June 15th. Sampling started 20 days later on July 5th and lasted until October 29th. Samples were taken weekly. For the first 2 weeks only 2 were taken each week, and the sample size increased to 4 per week thereafter due to having familiarized the routine. A total of 64 galls were sampled. In 2011, the first galls appeared on June 26th. Sampling started earlier, 11 days after gall emergence on July 7th. Initial sampling was also more frequent at 8 per week spread over 2 days, until September when sampling rate fell back to 4 per week. However, sampling in 2011 also ended earlier at the end of September due to adverse weather and personal illness. 84 galls were sampled in 2011.

Magnitude and trend of raw readings

In year 2010, oxygen levels detected in 60 galls were above 15kPa, constituting only mild hypoxia. There was no obvious change through the sampling period, although occasional readings as low as 3.3% did occur. The gall with 3.3% oxygen reading was one that suffered severe decomposition in the chamber, and the gallmaker did not survive. Only 2 galls showed oxygen levels below the average CO_2 P_C (9.5 kPa) on 2 separate

days, and 1 gall among them (the 3.3% measurement) showed oxygen level below the average activity P_C (3.6 kPa).

Gall oxygen levels measured in 2011 with the large probe however were much lower than those in 2010 with the small probe, at the same stage of gall development with regard to first gall emergence date. In 63 galls (out of 84) sampled over 20 days (out of 21), oxygen levels fell below the average CO_2 P_C. Among them, readings from 53 galls also fell below the average activity P_C, and such severe hypoxia occurred on 19 days. Highest readings taken in 2011 were in the range of 2010 data. Low readings were also observed less often as the 2011 season progressed and overall higher readings were more frequently seen (Figure 4-2).

Relationship with other factors

Following the AIC stepwise parameter selection, the last parameters remaining were: year, chamber penetration, gall diameter and internal chamber diameter (Table 4-2). The final model (Table 4-3) had adjusted R^2 of 0.67, suggesting a fairly strong correlation. Year was the most significant parameter in the regression, and the negative coefficient reflected lower readings in 2011. The effect of internal diameter was only nearly significant ($p = 0.053$). Penetration and gall diameter only slightly passed the significant threshold ($p = 0.041$ and 0.048 respectively). Both parameters showed positive regression coefficients, which was countary to the expectation for gall diameter.

Comparison with model expectations

Five galls from year 2010 were missing tissue layer thickness data, so only 59 galls from 2010 underwent modelling calculations. Overall speaking, modelled oxygen levels

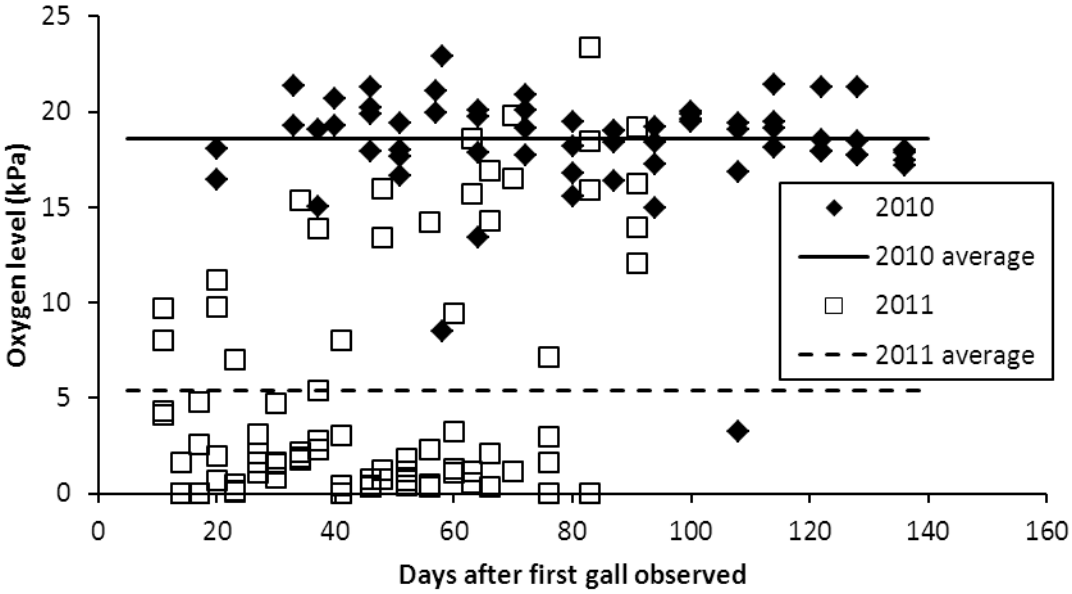

Figure 4-2: Directly measured oxygen level inside living galls. Difference between the two sampling years was apparent, with year 2011 showing low values more often at the same stage of gall development (estimated by appearance of the first galls of the season).

Table 4-2: Backwards model selection by AIC criteria. Regression was carried out on multiple parameters as predictors for gall oxygen level. The less important parameters in the model were removed. Calculated in R 2.12.0. The first and last steps are shown here. The complete results are in Appendix III due to excessive length.

Parameter removed	K[a]	RSS	AIC_C[b]	Δ_i[c]	w_i[d]
dryratio	11	3085.2	465.17	**0**	0.140
amass	11	3085.9	465.20	0.03	0.138
temperature	11	3090.0	465.39	0.22	0.125
date	11	3091.1	465.44	0.27	0.122
ppt	11	3091.7	465.47	0.30	0.120
afly	11	3101.8	465.93	0.76	0.096
diam	11	3114.2	466.51	1.34	0.071
phigh	11	3126.9	467.09	1.92	0.053
ghigh	11	3133.3	467.38	2.21	0.046
None	12	3085.2	467.54	2.37	0.043
penetrate	11	3149.9	468.14	2.97	0.032
idiam	11	3186.5	469.79	4.62	0.014
year	11	3423.5	479.17	14.00	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
None	4	3256.3	467.77	**0**	0.473
idiam	3	3343.0	469.55	1.777	0.194
diam	3	3347.1	469.73	1.957	0.178
penetrate	3	3353.3	470.00	2.227	0.155
year	3	4266.5	505.65	37.877	< 0.001

[a] Number of parameters

[b] AIC values with small sample size correction (AIC_C)

[c] Difference between the minimum AIC_C and the AIC_C achieved by removing the parameter in the current row. Δi of 0 indicates removing the current row's parameter leads to the minimum AIC_C.

[d] Akaike weight, interpreted as the probability that the current model is the best.

Table 4-3: Regression results of the last remaining model after AIC selection (N = 148).

Overall model	
F (4, 143)	74.08
p	< 0.001**
R^2	0.6745
Adjusted R^2	0.6654

Parameters	Regression coefficient	Standard error	t	p
Intercept	5.33	4.15	1.28	0.201
year	-9.00	1.35	-6.66	< 0.001**
penetrate	1.98	0.96	2.06	0.041*
diam	0.36	0.18	2.00	0.047*
idiam	5.61	2.87	1.95	0.053 n.s.

p value significance: n.s. $p < 0.1$, * $p < 0.05$, ** $p < 0.01$

in 2010 were similar to the mildly hypoxic field results, all being above 17 kPa. In 2011, modelled results showed more severe hypoxia and were comparable to the maximum values from field measurements in each day (Figure 4-3). Negative expected values (considered anoxia) were common before the cortex layer started its transition to the cytoplasm-poor state (July 19th). Alleviation of the initial severe hypoxia was also represented in the modelled results of 2011. For individual galls, modelled results did not match the field measurements well (summary in Table 4-4), and the modelled results were generally less hypoxic. The difference between modelled and measured values was greater in 2011 (Cohen's d, 2010 $d > 0.5$, 2011 $d > 0.8$). However, year 2011 data also showed stronger correlation (Pearson's $r = 0.284$, $t = 2.68$, $p = 0.009$) between field and modelled results than the 2010 results (Pearson's $r = 0.161$, $t = 1.23$, $p = 0.222$).

Discussion

Relation between field results, external parameters and modelling results

Sampling year was a major influence on oxygen level, as seen both on graph and from the linear regression results. Sampling in 2010 started later with respect to first gall appearance, so not being able to include the initial severe hypoxia in newly emerged, rapidly growing galls expected by the model (Chapter 3) may have contributed to the difference. However, while more severe hypoxia was detected in 2011, the expected transition from severe to mild hypoxia did not take place before the equivalent sampling period (same days after first gall emergence) in 2010. While the increase in maximum oxygen level was observed, low readings were commonly found during the same week or same day.

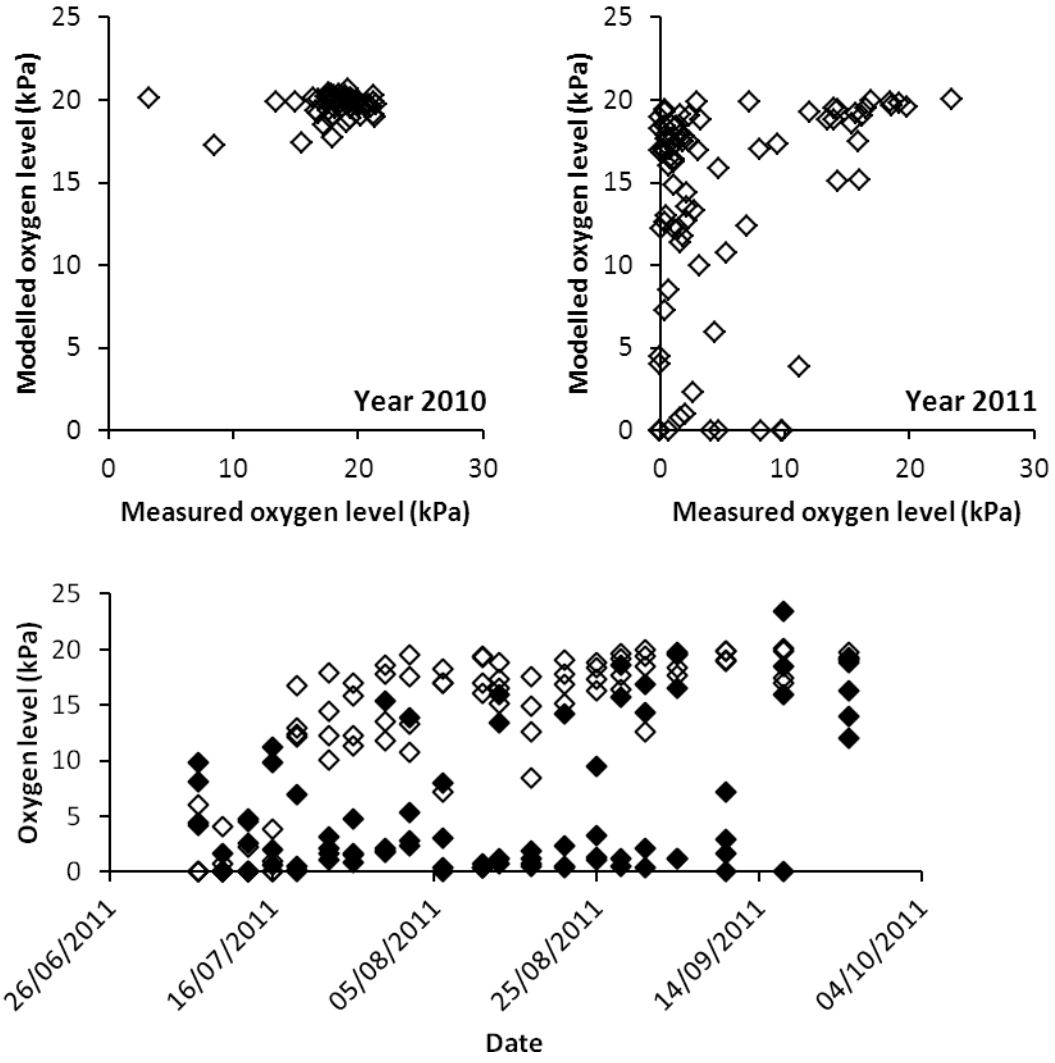

Figure 4-3: Correlation between field data and modelled oxygen levels. The difference between field and modelled results was smaller in 2010, but the correlation was also weaker, possibly due to the smaller variation in both field and modelled oxygen levels. The expected increase in oxygen level after initially severe hypoxia was represented in 2011 modelled oxygen levels (empty markers).

Table 4-4: Testing for difference between modelled and measured oxygen levels.

	Year 2010 (n = 59)	Year 2011 (n = 84)
Difference[a]	1.55 ± 3.02	8.34 ± 7.82
Paired t-test[b]	t = 3.93, p < 0.01	t= 9.78, p < 0.01
Effect size[c]	0.51	1.07
Correlation[d]	r = 0.161	r = 0.284
Correlation test	t = 1.23, p = 0.222	t = 2.68, p = 0.009

[a] Modelled result – field measurement result, mean ± SD

[b] H_0: mean difference = 0; H_A: mean difference > 0

[c] Cohen's d for paired t-tests: mean of differences/standard deviation of differences

[d] Pearson's r

Gall diameter was expected to negatively influence oxygen level due to the increase of diffusion distance and volume of respiring tissue. However, the gradient term for diameter in the regression was positive, suggesting larger galls in the dataset had higher oxygen levels. This may have been due to correlations with year and date. Year 2010 had larger galls (Figure 4-4A) and higher oxygen level, and galls later in development (larger size) also showed higher oxygen level in 2011. These effects may have been stronger than the expected effect by gall diameter, so that large galls were associated with high oxygen level in the regression.

Internal diameter and successful chamber penetration by the probe were likely correlated with each other, because larger chambers were more easily targeted by the probe. They would also be correlated with the increase in oxygen level detected with development time, due to the increase of chamber size with larval development. Therefore, high oxygen levels later in development would be correlated with larger chambers that were more easily penetrated. In addition, high oxygen level in larger chamber was an expectation of the model in Chapter 3, possibly due to the large chamber's contribution to the surface area over which diffusion may occur. Probes that fail to penetrate the chamber essentially stay inside a smaller "chamber" represented by the space at the tip of the close-ended tunnel, making the expectation that successful penetration has similar effects to larger chamber size more reasonable.

Among the modelling results, weaker correlation between field measurement and calculated values in 2010 seems counterintuitive at first. However, the 2011 data showed greater variation with similar trends of increasing oxygen levels later in the season, while field and calculated values in 2010 were only mildly hypoxic and showed little variation

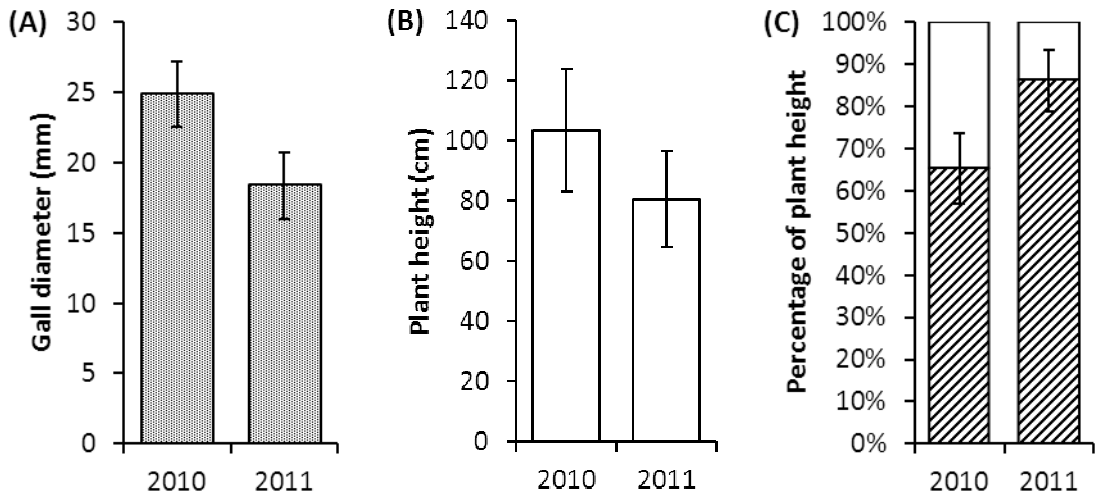

Figure 4-4: Difference of host plant and gall growth between years. In year 2010 (n = 36), Galls were larger (part A, two-tailed $t = 13.54$, $p < 2.2\times10^{-16}$) and host plants were taller (part B, one-tailed $t = 5.17$, $p = 1.670\times10^{-6}$) in 2010 (n = 36) than in 2011 (n = 72), between 20 and 91 days after first gall appearance. The time period was chosen due to data availability in both years, covering early-mid July to mid-late September and included blooming of host flowers. Stem height below gall (striped bar, part C) contributed more to total plant height in year 2011, suggesting plants were older at oviposition (one-tailed t-test after arcsine transformation, $t = 13.18$, $p < 2.2\times10^{-16}$). Error bars represent standard deviation.

(Figure 4-3). The more apparent trend may have been represented better by the correlation. The less hypoxic modelled results suggest that parameters causing the hypoxia were underestimated in the model. One possibility is the underestimation of plant respiration, due to the removal of sample tissue from the plant (despite efforts made to start measurement as soon as possible and to reduce dehydration). In addition, the difference of modelling accuracy between years indicates some parameters showed between-year differences in the field. These parameters may be the ones taken as being uniform in the calculation (respiration, diffusion and tissue density values) or ones not taken into account by the model. The uniform parameters were mostly estimated in 2011 (except animal respiration, the least influential parameter in the sensitivity analysis), so their difference between years could not be evaluated. Subsequent discussion would focus on influences from outside the model.

Potential association of 2011 hypoxia with plant and gallmaker health

The difference between oxygen level measurements in 2010 and 2011 was not fully explained by gall development, and as tested in the methods section, change of probe should not have led to such significant and consistent differences. Difference between these two years was not limited to oxygen level. An obvious difference observed while collecting samples in the field was that galls found in 2011 were smaller (Figure 4-4A). Survival of gall inhabitants was also poor in 2011, not only for *E. solidaginis* itself, but also for the parasitic wasp *Eurytoma obtusiventris* (Figure 4-5). Unlike *Eurytoma gigantea* which consumes the entire *E. soldiaginis* larva, *Eurytoma obtusiventris* larva causes premature pupation of the host larva, and is found inside the empty *E. solidaginis* pupa. Therefore, presence of *Eurytoma obtusiventris* would indicate the *E. solidaginis*

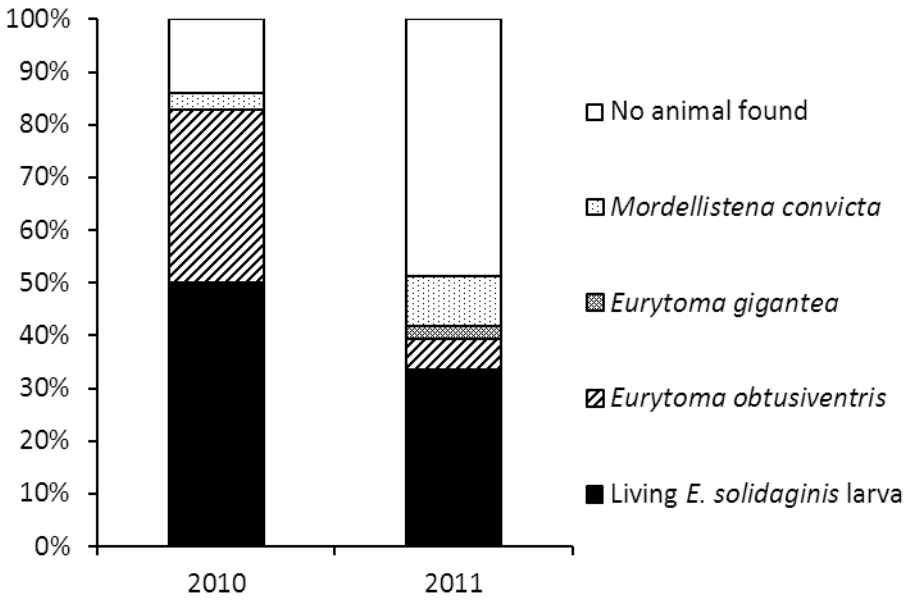

Figure 4-5: Difference of gall content between years. Significant difference ($\chi^2 = 34.11$, df = 4, $p < 0.001$) was found between the content of galls taken for direct oxygen measurements in years 2010 (n = 64) and 2011 (n = 84). No dead *E. solidaginis* visible to the naked eye were found in either year. *Eurytoma obtusiventris* parasitizes *E. solidaginis* larvae and are found inside empty *E. solidaginis* pupae, therefore low *Eurytoma obtusiventris* occurrence in 2011 may be additional evidence for reduced *E. solidaginis* performance.

larva had once been developing healthily. Taking *Eurytoma obtusiventris* survival into account suggested that potential *E. solidaginis* survival in 2011 could be as low as 1/2 of 2010 survival. Small gall size should also lead to greater mortality from *Eurytoma gigantea* attacks, but *Eurytoma gigantea* was nearly absent at the research site in both years. Instead, the greatest source of mortality in 2011 was finding empty galls, indicating the larva died very early in development. Poor gallmaker survival seemed like a regional instead of site-specific event. *E. solidaginis* galls were also collected for a teaching lab in both years. In 2010, over 50% of galls collected from Short Hills Provincial Park contained living *E. solidaginis* larvae, which was comparable to survival in galls used for field measurements. In 2011, about 1/3 of galls collected on the Niagara Peninsula contained living larvae, while 2/3 of galls from Hamilton or Peterborough, ON (collected September 26, 2011) did. Weather difference was the first to come to mind. To further investigate the late gall emergence observed in 2011, earliest possible dates for *E. solidaginis* pupation and emergence were estimated by matching daily temperature data recorded by the Vineland RCS (Reference Climate Station, WMO 71171, NCDIA) to temperature requirements determined by past research. Compared to the St. Catharines Airport station used previously, the Vineland station has mean daily temperature data available, whereas the Airport station was used for weather descriptions because it was closer to campus and should therefore accurately reflect campus weather more often. *E. solidaginis* pupation requires an average mean temperature of above 44°F (6.6°C) for 2 weeks, while emergence requires an average mean temperature of 59°F (15°C) for 10 days (Uhler 1951). Weather data showed that temperature permitting pupation and emergence arrived 18 and 3 days late respectively in 2011 compared to 2010 (Figure 4-

6). Actual observations of oviposition and gall production also occurred later in 2011 by 6 and 11 days respectively. The criteria used were estimated in Varna, New York, which is approximately 1° south of St. Catharines in latitude. St. Catharines and Varna belong to plant hardiness zones 6b and 5b respectively (Natural Resources Canada, 2000; United States Department of Agriculture, 2012), indicating that minimum winter temperature in St. Catharines is higher, likely due to moderating effects from two Great Lakes bordering the Niagara Peninsula. Considering different adaptations to local climate by *E. solidaginis*, temperature requirements from one location may not accurately predict dates in another. For studies with greater focus on phenology, location-specific criteria may have to be established.

In the wild, galls that appear later were observed to reach smaller final sizes (Weis and Abrahamson 1985). A subsequent greenhouse experiment found that increasing plant age at oviposition negatively affected final gall size, possibly reflecting a loss of reactivity to gallmaker stimuli as the plant ages (Weis and Abrahamson 1985). Late oviposition also leads to fewer galls forming and fewer larvae surviving until adulthood, even when mortality due to the gall-size-dependent parasitoid *Eurytoma gigantea* did not differ between oviposition periods (Horner *et al.* 1999). Galls found in 2011 were indeed smaller and showed poor survival, but were they really produced on older plants due to late adult emergence and oviposition?

E. solidaginis larva moves from the egg into the apical meristem to induce gall growth (Uhler, 1951). The meristem is elevated by subsequent stem growth, while the gall remains at its initial location. Hence gall height is an indicator for plant height at oviposition (assuming a constant time from oviposition to gall induction), and stem

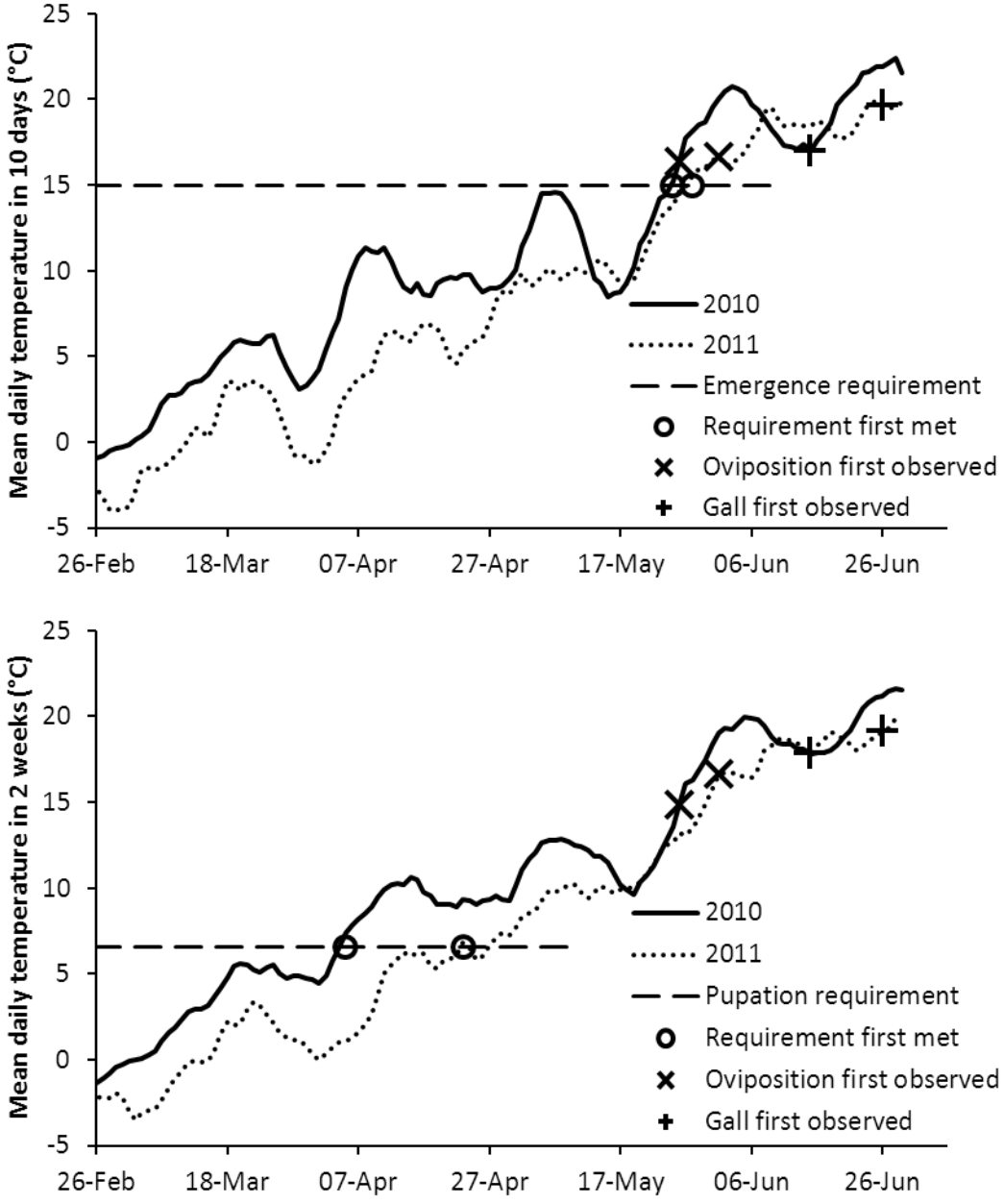

Figure 4-6: Temperature requirements for *E. solidaginis* pupation and emergence. According to criteria estimated by Uhler (1951), temperature requirements that permit pupation and emergence were fulfilled later in 2011 than in 2010, possibly leading to delayed oviposition and gall production. Temperature data were recorded at the Vineland RCS (WMO ID: 71171, retrieved via the NCDIA).

length above the gall represents plant growth that occurred after oviposition. Late oviposition means more time would be available for plant growth before oviposition, so that height below the gall should represent a greater proportion of total plant height. In addition, host plant growth rate decreases with age (Horner *et al.*, 1999), reducing post-oviposition growth even more. The ratio of gall height to total plant height was higher in 2011 (Figure 4-4C), suggesting oviposition was indeed late in 2011 compared to 2010. Plants were also taller in 2010 (Figure 4-4B), suggesting a greater overall plant growth rate. So, severe hypoxia detected in year 2011 coincided with late oviposition, poor growth of host plant and the gall itself, and poor survival of *E. solidaginis* larvae. Is it then possible to explain the hypoxia as a cause or result of the other observations?

A large number of data points showed gall oxygen levels below the larva's P_Cs in 2011. This implies reduced metabolism and feeding activity in the larva, which would likely lead to slow growth (if at all) and poor survival during a life stage dedicated to accumulation of biomass. In addition, the reduction of metabolism in hypoxia may have been insufficient for energy conservation under high summer temperatures. Even during the winter diapause, cold temperature and low metabolism increased the larva's survival (Irwin and Lee, 2003), so the effect on survival would likely be greater during the summer. On the other hand, what could poor plant growth have to do with hypoxia? Weather is considered again as a possible influence. While early spring of 2011 was colder than 2010, the region surrounding the study site also saw a warm and dry summer. In daily weather data gathered for the regression (see methods section, from the NCDIA and personal observation), precipitation occurred on 11, 8 and 10 days in June, July and August of 2010 respectively, compared to 8, 4 and 7 days in 2011. Detailed data on the

daily amount of precipitation were available from the Vineland RCS (WMO ID 71171), located approximately 15 km northwest of Brock University. During the three weeks after first gall appearance, which cover the crucial period of initial gall growth, year 2010 (15th June – 5th July) received 24.8 mm of precipitation as opposed to 4.7 mm in 2011 (26th June - 16th July). June and July in total saw 128.4 and 64.6 mm of precipitation respectively in 2010, compared to 54.9 and 38.3 mm in 2011. This also means June of 2010 received more than the long-term average precipitation of 81.5 and 71.8 mm (Canadian Climate Normals 1971-2000 at Vineland Station, located less than 400 meters south of the current RCS, retrieved again from NCDIA), while both June and July of 2011 received less. In terms of temperature, June-August in both years were warmer than the 1971-2000 normals, expressed by monthly averages of daily mean, maximum and minimum temperature. Only in July was 2011 (mean 24.2°C, max 29.4°C, min 18.9°C) warmer than 2010 (mean 23.4°C, max 28.5°C, min 18.3°C).

Drought itself is known to reduce gall size and larva performance via gall desiccation and slow plant growth under stress (Sumerford *et al.,* 2000). As for the relation with gall oxygen level, gas exchange in plants can be affected by environmental conditions. Plant surface is covered with a layer of waxy cuticle that reduces water loss and diffusion in general, and gas exchange takes place mainly through stomata which are connected to a system of intercellular spaces (Salisbury and Ross, 1992). High temperature may stimulate or suppress the extent of stomatal opening, due to the need for cooling by transpiration or rising CO_2 level at an elevated respiration rate respectively. Water stress reduces stomatal opening to limit water loss via transpiration (Salisbury and Ross, 1992). When response to water stress was simulated with abscisic acid (ABA),

stomatal opening at the same temperature is reduced as a trade-off between water conservation and cooling (Reynolds-Henne *et al.,* 2010). Stomata closure should also reduce the amount of O_2 exchange through stomata. The cuticle's permeability to CO_2 and water vapour also decreases under water stress (Boyer *et al.,* 1997). If the same applies to oxygen gas, then reduced precipitation may have also reduced oxygen exchange through the cuticle. While temperatures were comparable in the two years' summers (which were higher than the area average), plants in 2010 were exposed to more than the area's average precipitation, while the 2011 plants were likely under more severe water stress. Therefore, host plants in 2011 were more likely to show reduced stomata opening and reduced cuticle permeability, which may have limited oxygen availability to gall tissue and the larva. In addition to previously known effects of drought on mortality, reduced oxygen availability due to water stress and increased metabolism due to high temperature may have been detrimental for the larva during the 2011 growth season.

However, temperature and precipitation were not found to be important parameters in the regression with oxygen level. The AIC model selection (Table 4-2) was repeated with the more detailed precipitation data (sum of the amount of precipitation in mm, instead of days with precipitation, on sampling day and in the previous week) from Vineland RCS, but the final model stayed the same, and the effect of precipitation was not significant. Besides interaction between these two parameters and interference from other field conditions, sampling bias may also have been a confounding factor. Field measurements were typically carried out just before noon on sunny days to keep electronic equipments away from rain. This would have prevented samples being taken soon after precipitation, and the data would have predominantly represented dry and

warm conditions. In addition, temperature inside the gall shows more extreme variation than air temperature does when under direct sunlight (Layne, 1993), so a regression with air temperature may have shown weaker relationship. Lastly, host genotypes may respond differently to drought and show variable influence on gallmaker fitness (Horner and Abrahamson, 1999). If the influence on fitness involves oxygen level changes, then responses from uncontrolled host genotypes may have been a confounding factor.

In summary, weather conditions of 2011 spring and summer caused *E. solidaginis* to oviposit on older host plants which subsequently suffered poor growth due to drought in the summer. The lasting hypoxia observed may have been driven by plant response to drought, and was not necessarily directly related to plant growth. Host age, host growth and gall desiccation were all known as sources of larval mortality. Since these factors were likely all in effect during the 2011 growth season, it is impossible to determine whether hypoxia also affected larval fitness. Further experiments are required to determine how *E. solidaginis* larva may be affected by prolonged severe hypoxia.

Importance of larval mortality after gall formation

Presence of *E. solidaginis* galls reduces the plant's energy allocation to photosynthetic and reproductive organs (Hartnett and Abrahamson, 1979), so defense against the gallmaker should be adaptive for the host plant. Certain host defenses are able to kill the larva before gall formation (Abrahamson and Weis, 1997), but galls may also form before or despite larval death. Is it still beneficial for the host to kill the gallmaker despite not being able to stop gall formation? Stinner and Abrahamson (1979) assessed the energy budget of *E. solidaginis* galls through biomass and respirometry, and found

that galls attacked by parasitoids contain less energy than galls with healthy gallmakers. In the case of *Eurytoma obtusiventris* attack, the affected *E. solidaginis* larva stops feeding prematurely, consuming less biomass in total (Abrahamson and Weis, 1997). The nutritive layer may also be replenished by cell division due to larval stimulus (Weis *et al.*, 1989), and cessation of feeding would stop the stimulus and therefore reduce energy allocation to gall growth. Larval death due to other causes during the growth season may also have similar effects in reducing the gall's energy burden on the host.

In addition, biomass consumed by the larva is lost to the plant, but biomass remaining in the gall might not be lost completely. Underground rhizomes are important in resource storage and support of new ramets. Nutrient in aging leaves can be translocated to underground organs in autumn (Abrahamson and Weis, 1997). If the same applies to nutritive gall tissue, then stopping larval feeding would allow more resources to be retrieved from the dying gall. However, the host plant may respond to gallmakers by isolating affected ramets through deterioration of rhizome connections, in order to reduce the resource drain on healthy ramets (McCrea and Abrahamson, 1985). This would make resource "recycling" late in the season unable to benefit future ramets from the main rhizome network. Still, if isolated sections of rhizomes survive to produce new ramets, nutrients saved by early larval death could be put to use.

Possibilities of gallmaker or host influence on gall oxygen level

So far, development of the cytoplasm-poor cortex layer and the host plant's drought response have been proposed as major influences to the oxygen level to which *E. solidaginis* larva is exposed. The former may benefit the larva by increasing oxygen

availability, while the latter may restrict oxygen availability, therefore potentially contributing to early larval death and reducing host resource lost to the gallmaker. Previously in this thesis, both processes have been proposed as simple extensions of healthy plant physiology. Is it however possible for either the host or the gallmaker to exert at least some influence on these processes for their own benefit?

Pith autolysis, proposed as the basis of the cytoplasm-poor cortex, is regulated by ethylene production (Carr and Jaffe, 1995). If the larva is able to produce ethylene or induce ethylene production by the host plant, then it would be able to affect the transition from fresh cortex to the cytoplasm-poor state. However, if ethylene is produced by the larva itself, then ethylene diffusing outwards from the larva would affect the nutritive layer first. If cytoplasm reduction occurs in the nutritive layer, food supply for the larva would be depleted. Hence in order for the larva to affect cytoplasm reduction while maintaining the gall as a food supply, there must be a process in the nutritive layer that offsets the effect of ethylene from the larva. Or else, ethylene production by the host plant itself needs to be manipulated by the larva. Of course, if cytoplasm reduction due to plant physiology alone enables sufficient oxygen supply to the larva, there would be no selection for the larva to further stimulate the process. To study this problem further, artificial induction of gall-like swellings may be useful. Comparing the development of galls from other gallmakers on hosts that undergo pith autolysis may also be useful.

Abscisic acid (ABA) is an important plant hormone in signalling stress, including drought (Zhang *et al.*, 2006). It has been used in experiments investigating drought response as a substitute for actually growing plants in drought (Reynolds-Henne *et al.*, 2010). Restricting drought response via suppression of ABA production should be

technically possible, but whether larval mutation can produce such characteristics is another matter. If *E. solidaginis* is capable of such manipulation, it certainly was not completely effective in 2011. Drought also contributes to larval mortality through other means including desiccation and reduced host growth (Sumerford *et al.*, 2000), so simply opening stomata will make desiccation more severe and nevertheless lead to larval mortality. If these other factors cause larval mortality earlier than hypoxia, manipulating the drought response would be even less adaptive. In addition, the adaptive value of proper drought response for the plant means plant genotypes vulnerable to such influence will be strongly selected against. So it would seem that the larva is powerless against hypoxia caused by the plant's drought response. On the other hand, elevating drought response to kill the larva also brings in the tradeoff associated with stomata closure at high temperature (Reynolds-Henne *et al.*, 2010). Given that detrimental effect from *E. solidaginis* galls tends to be restricted to individual ramets instead of the whole clone (Abrahamson and Weis, 1997), fitness gain from such drastic anti-gallmaker characters may not be sufficient to overcome the tradeoff.

Comparison to the other gall known to be hypoxic

As mentioned in the introduction chapter, Haiden *et al.* (2012) also identified hypoxia in an insect gall, induced by the wasp *Trichilogaster acaciaelongifoliae* on *Acacia longifolia.* Hypoxia found in gall tissue was more severe (below 5%), in contrast to the mild hypoxia above 15 kPa that lasts for most of the growth season, which is expected from modelling and detected in year 2010 for *E. solidaginis*. Oxygen level in the larval chamber was even lower at about 2.4%. *T. acaciaelongifoliae* galls may reach sizes comparable to *E. solidaginis* galls (20 mm diameter), but do not have a thickened

cortex layer, and development into the cytoplasm-poor state was not mentioned (Dorchin *et al.,* 2009). This may be additional evidence that cytoplasm reduction in *E. solidaginis* galls served to alleviate hypoxia. *T. acaciaelongifoliae* is subject to parasitism, with shallow chambers being at greater risk (Manongi and Hoffmann, 1995). A tissue layer analogous to the cortex in *E. solidaginis* galls should contribute to defense against parasites. Its absence may indicate balancing selection pressure, such as factors analogous to bird predation on *E. solidaginis* or increased host defense due to extra host nutritent cost, or differences in host tissue that give rise to the gall. Although *E. solidaginis* eggs are laid in leaf buds, the gall itself develops as a swelling of the stem, and we proposed the cytoplasm reduction as an extension of pith autolysis that occur in stems. *T. acaciaelongifoliae* galls in contrast develop from reproductive and vegetative bud tissue (Dorchin *et al.*, 2009). If bud tissue lacks the mechanism for pith autolysis, *T. acaciaelongifoliae* galls may be unable to develop a thick, cytoplasm-poor tissue layer.

Chapter summary

Direct measurements of gall oxygen level in years 2010 and 2011 showed discrepancy between years not fully explained by the stage of development. Results from 2010 were mildly hypoxic as expected by the model in Chapter 3 for mature galls, while results from 2011 showed severe hypoxia lasting beyond the expected period of severe hypoxia in young galls. Year 2011 also saw poor plant growth and increased larval mortality, which coincided with more extreme weather conditions, with cold spring and warm, dry summer. In response to the water stress, changes in the plant's stomata and cuticle may have reduced gas exchange, which affects oxygen availability. High temperature would increase plant and larval metabolism, so that both the extent and

consequences of hypoxia became more severe. Late oviposition due to cold spring and drought in the summer may also have reduced larval performance, and distinguishing the effect of hypoxia from them requires further study.

Chapter 5: Summary and potential for further studies

Summary of observations and explanations

The individual chapters' main findings were:

- Chapter 2: *Eurosta solidaginis* larva showed a depression in metabolic rate when oxygen levels fell to 9.5 kPa on average, and became inactive at on average 3.6 kPa. These P_C values were not extremely low by insect standards, suggesting *E. solidaginis* larvae were not adapted to prolonged and severe hypoxia during the growth season.
- Chapter 3: During the "development" of a mathematically modelled gall, oxygen levels as low as the numbers above lasted for less than 2-3 weeks. The short time period was consistent with the lack of extremely low P_C.
- Chapter 4: In year 2010, directly measured oxygen levels in wild galls were mildly hypoxic and generally supported model expectations. However, in year 2011, the galls showed much more severe hypoxia than expected. Poor plant growth and low gallmaker survival were also observed in 2011. Low precipitation during the summer was suggested as a cause for the severe hypoxia, and also leads to slow plant growth and larval mortality. The effect of hypoxia on *E. solidaginis* fitness requires further study.

Therefore the current answer to the main question is: Hypoxia does occur in galls produced by *Eurosta solidaginis*, in the sense that oxygen level inside galls fell below the atmospheric level. The larva should tolerate this oxygen level without the need for metabolic depression or reduced activity during most of the growth season. More severe

hypoxia observed in year 2011 could potentially have reduced larval fitness, and high larval mortality was indeed observed in the same year. However, since the observations coincided with detrimental weather during growth that is known to cause larval death by itself, there is no evidence that gallmaker fitness was directly affected by hypoxia.

Development of the gall's cortex layer into the eventual cytoplasm-poor state should be a main influence on the larva's oxygen availability. This process reduces the gall's oxygen consumption and increases its permeability to gases, therefore allowing more oxygen to reach the larva. After the gall reaches full size and before cytoplasm reduction starts, a short period of more severe hypoxia is expected in the modelling results. It is possible that slow growth in young larva coincides with this period, so that high metabolic rate is not required during the most severe hypoxia. Early hypoxia may also be a factor in mortality of young larvae. However, the occurrence of this period could not be confirmed by field measurement data, since sampling in 2010 started late, and data in 2011 may have been affected by the year's extreme weather. Other potential influences on gall oxygen level include gall size, central chamber size and the host plant's stomata control under water stress.

Limitations in further adaptation regarding gall oxygen level

Given the goal of studying potential gall hypoxia is to explore the possibility of adaptation through gall modification, processes suggested as influences on gall oxygen level had been discussed further in the respective chapters. Gall formation likely involves only manipulating plant genes that serve other functions in the healthy plant, since rendering these genes nonresponsive to gallmaker stimuli would lead to detrimental pleiotropic effects that outweigh the benefit of increased defense against gallmakers

(Abrahamson and Weis, 1997). It seems that potential adaptations involving gall oxygen level are also under such constraints. Examples are gall size which is under selection by predators, and stomata control that is important in balancing gas exchange and water loss. Chamber size on the other hand is limited by the lack of variation in nutritive layer thickness, and also by the rate of feeding the larva is capable of. Thickness of the cytoplasm-poor cortex may be constrained by limitations in both gall size and nutritive layer thickness. For *E. solidaginis*, it may have been easier to adapt to the largely mild hypoxia in its gall through physiology, instead of through modification of the gall phenotype.

On the other hand, the host plant may also have limited ability to evolve resistances against the gallmaker through hypoxia. More severe hypoxia could be achieved by thicker and more metabolically active gall tissue, which defeats the purpose of reducing energy loss due to the gallmaker in the first place. Due to the highly clonal growth pattern, the impact of *E. solidaginis* on host fitness on the genotypic level is generally regarded as low, and the benefit of added defence through hypoxia may not be enough to overcome the disadvantages.

Improvements to methods

Wild galls were used as research subjects. This decision was made to collect data with all potential variations under natural condition, such as poor gall growth and gallmaker survival observed in 2011. However, these variations also interfere with analyses, and future studies with clear hypotheses and more experimental elements should consider using controlled conditions. Specifically, in chapter 4 we discussed the possibility of host and plant development affecting gas exchange inside the gall. In a

controlled environment factors involved could be more easily manipulated. The entire *E. solidaginis* life cycle has been studied in laboratory settings (Uhler, 1951). Host plant genotypes are also easily controlled via clonal propagation of rhizomes (e.g. Craig *et al.* 2007). Such experiments would likely require large agricultural facilities.

A source of doubt in the field measurement results was the change of oxygen probe used in field sampling between 2010 and 2011. Fortunately follow-up experiments found no difference between probes. One factor in the decision of probe change was that bringing the long, thin, fragile-looking FOXY-AF probe into the field repeatedly opens up opportunities for probe damage, which would not only be costly but also affects subsequent readings. The thicker FOXY-R was sturdier. A controlled setting would be less demanding for instruments in terms of field condition, transport distance, equipment (e.g. power supply) availability and time limit, so more delicate and potentially less invasive methods could be used.

Estimation of gall tissue diffusion coefficients failed to fully satisfy the assumption demanded by the calculation. During the planning stage a method involving finite element analysis was considered (Lammertyn *et al.*, 2001), but was abandoned due to a lack of understanding of its theoretical background to replicate the method with available software. That method takes plant respiration into account, so chemical inhibition is not required, but plant respiration still needs to be measured. If further studies are a possibility, similar methods that require little sample preparation should be considered for estimating diffusion coefficients. Simple visual estimation of tissue boundaries could also have affected sample quality by including more than one type of tissue in each sample. Determination of tissue layer boundaries has previously been done under microscope

(Weis *et al.*, 1989), and more precise cutting methods may be needed to extract required tissue layers. In a controlled setting, it may be desirable to identify host and gallmaker genotypes that produce larger galls to acquire more material.

Possibilities for further research

A future research possibility is investigating whether cytoplasm reduction of the cortex layer is affected by the larva. This process is the best candidate as a potential adaptation through the gall phenotype to alleviate hypoxia, because it also reduces the host's energy cost and therefore does not select for better host defense against the gallmaker (Weis *et al.*, 1989). It may be useful to make comparisons with other gallmakers, including those on goldenrods. Moths in genera *Gnorimoschema* and *Epiblema* produce elliptical or spindle-shaped galls easily distinguished from *E. solidaginis* galls. These galls have larger chambers and smaller diameter, so gas exchange could progress differently even in freshly-formed galls (if similar developmental stages are present). Whatever the subject might be, if their galls contain a layer of significant thickness that is not fed on by the gallmaker, then the layer should be a suitable focus.

Another possible direction is the details of gas diffusion in the gall. In Chapter 3, a simple model assuming uniform characteristics within each tissue layer was used. Further studies may deal with the characteristics of the stomata, intercellular spaces, or spaces in the cytoplasm-poor cortex layer, as analogues to studies in egg shell porosity (e.g. Wangensteen and Rahn, 1971) in which other models were developed. It may also be useful to consider the central chamber's similarities with the plastron in diving insects. The chamber would be an analogue to the bubble around the insect body, surrounded by the nutritive layer with high water content. The effectiveness of the plastron is influenced

by surface area (Flynn and Bush, 2008), like the expected effect of chamber size on the Chapter 3 model.

The exact nature of the CO_2 and activity P_Cs may also be important in further physiology studies. The distinction between aerobic and anaerobic oxyconformity is the activation of anaerobic processes (Pörtner and Grieshaber, 1993). Freezing causes tissue-level anoxia (Morin *et al.,* 2005) during which *E. solidaginis* larvae supplement energy production with anaerobic glycolysis (Storey and Storey, 1985). Therefore, analyses for anaerobic end products, previously used in studies of freezing tolerance (e.g. Storey and Storey, 1985), may be useful for the detection of anaerobic metabolism. The possibility that metabolic depression was time- instead of oxygen-dependent could be investigated by interrupting the progressive hypoxia exposure so that oxygen level remained at a certain level (different between treatments), and monitoring the change of metabolic rate in the absence of more severe hypoxia. In addition, more sensitive equipments may be needed to compensate for the larva's lack of mobility.

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Appendices

Appendix I: R script used in estimation of CO_2 P_C.

The script runs the procedure that determines the critical point in a folder of .csv files (and a .csv file containing the file names of data files), based on Yeager and Ultsch (1989)'s BASIC program. The script can be pasted into the R window directly or into R Commander (*rcmdr*), and has only been used in R 2.12.0 or above. Comments start with the "#" sign.

```
    # Based on: Physiological Regulation and Conformation: A BASIC Program for the Determination of Critical Points
    # Dorian P. Yeager and Gordon R. Ultsch
    # Physiological Zoology, Vol. 62, No. 4 (Jul. - Aug., 1989), pp. 888-907
    # Fits two linear regressions across a series by changing the point of division between two parts of the series one by one.
    # The fit that minimizes total RSS (residual sum of squares) of the two lines is chosen as the best division.
    # The intersection of the two lines has O2 = Pcrit.
    # Developed to analyze .txt files saved from Acqknowledge 3.8.1, with data channels representing: O2 reading, CO2 reading, temperature reading, activity reading, and root mean square (RMS) of activity. RMS was calculated as a visual indicator of activity level, but activity data were analyzed with other calculations.

    # Defines source and save path. By default, the file list is named "files.csv".
    path <- "source folder path using "/" symbol, e.g. E:/2fittest/"
    namecsv <- paste(path, "files.csv", sep = "")
    savepath <- paste(path, "saved/", sep = "")

    # The source folder should also contain a .csv file with names of files to be processed, one in each row.  By default, the file list is expected to have a header row marked "NAME". R can be programmed to read file names automatically, but that was not done here.
    Filenames <- read.table(namecsv, header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE, fill=TRUE)
    Filenames$sourcepaths <- paste(path, Filenames$NAME, ".txt", sep="")
    Filenames$savepaths <- paste(savepath, Filenames$NAME, ".csv", sep="")
```

```
     Filenames$graphpaths <- paste(savepath, Filenames$NAME, ".pdf",
sep="")
     Filenames$pngpaths <- paste(savepath, Filenames$NAME, ".png",
sep="")

     # Create the folder to save subsequent files (trimmed data file and
graphs)
     dir.create(savepath)

     # Start counting the number of files processed
     filecount <- 1

     # Start the vectors used to save the final key readings
     interrow <- c(0)
     sepO2 <- c(0)
     interO2 <- c(0)
     interCO2 <- c(0)
     line1slope <- c(0)
     line1intercept <- c(0)
     line1r2 <- c(0)
     line2slope <- c(0)
     line2intercept <- c(0)
     line2r2 <- c(0)

     # Start looping through the folder, read files
     for(i in 1:length(Filenames$NAME))
      {

            # Input data with FILL=TRUE and HEADER=FALSE, in case some
columns have empty spaces at the end due to recording error.
AcqKnowledge .txt files by default have no header, so HEADER=FALSE is
specified.
            Dataset <- read.table(Filenames$sourcepaths[filecount],
header=FALSE, sep="", na.strings="NA", dec=".", strip.white=TRUE,
fill=TRUE)

            # Give column names
            colnames(Dataset) <- c("O2","CO2","TEMP","ACT","RMS")

            # Correct for flow delays: 41 rows (33 sec) between
activity/RMS/temperature probes (electronic, assume no delay) and CO2
analyzer, and 11 rows (9 sec) between CO2 and O2 analyzers, calculated
from flowrate and tube diameter and length
            delay41 <- c(1:41)/c(1:41)
            delay11 <- c(1:11)/c(1:11)
            delayO2 <- c(delay41, delay11, Dataset$O2)
            delayCO2 <- c(delay41, Dataset$CO2, delay11)
            delayTEMP <- c(Dataset$TEMP, delay41, delay11)
```

```
            delayACT <- c(Dataset$ACT, delay41, delay11)
            delayRMS <- c(Dataset$RMS, delay41, delay11)
            delaytotal <- length(Dataset$O2) - 52
            Dataset <- data.frame(delayO2[53:delaytotal],
delayCO2[53:delaytotal], delayTEMP[53:delaytotal],
delayACT[53:delaytotal], delayRMS[53:delaytotal])

            # Give column names again
            colnames(Dataset) <- c("O2","CO2","TEMP","ACT","RMS")

            # Correct for min O2 (0.3% corresponding to expected 99.7%
pure nitrogen), ymincor is what O2% the recorded O2 is designated as
            # First establish the straight line between (xmin, ymincor)
and (xmax, ymax)
            minrow <- which.min(Dataset$O2)
            maxrow <- which.max(Dataset$O2)
            xmin <- Dataset$O2[minrow]
            ymincor <- 0.3
            xymax <- Dataset$O2[maxrow]

            b <- (xymax*ymincor - xmin*xymax)/(xymax-xmin)
            a <- (xymax - b)/xymax

            # Then calculate a corrected value for all O2, designate
the new vector as O2 and the old as O2original

            Dataset$O2original <- Dataset$O2
            Dataset$O2 <- Dataset$O2 * a + b

            # Only use the period after O2 first decreases to below 20
and before corrected O2 first drops to below 0.6, the gap between first
below 0.6 and rise above 0.6 is at least 10 readings long to reduce the
possibility of including extreme sensor fluctuations
            Dataset$rows <- c(1:length(Dataset$O2))
            trim1 <- 20
            trim2 <- 0.6
            Trimset <- subset(Dataset,Dataset$O2 <= trim1)
            Trimset <- subset(Trimset,Trimset$O2 >= trim2)
            Trimmedset <- Trimset
            trimcount <- 100 #ignore the first 100 rows in Trimset as
potential beginning of the gap
            gapsize <- 10
                  for (i in 1:(length(Trimset$O2)-101))
                        {
                              if ((Trimset$rows[trimcount+1] -
Trimset$rows[trimcount]) > gapsize)
                                    {
```

```
                                    Trimmedset <- subset(Trimset,
Trimset$rows <= Trimset$rows[trimcount])
                                    break
                              }else
                              if (trimcount < length(Trimset$O2))
                              {
                                    trimcount <- trimcount+1
                              }
                        }

            # Convert corrected O2 percentage to kPa, after saving the
corrected O2 percent as O2percent
            Trimmedset$O2percent <- Trimmedset$O2
            Trimmedset$O2 <- Trimmedset$O2*Filenames$KPA[filecount]/100

            # Save the part left by trimming in csv file
            Trimmedset$rows<-NULL
            write.table(Trimmedset, Filenames$savepaths[filecount],
sep=",", col.names=TRUE, row.names=FALSE, quote=TRUE, na="NA")

            # START using the trimmed contents

            # Sort the dataset by increasing O2 level
            Trimmedset <- Trimmedset[order(Trimmedset$O2),]

            # Define how many datapoints to ignore as potential
intersection from both ends
            ignore <- 100
            loop <- length(Trimmedset$CO2)-2*ignore

            # Start counting datapoint
            count <- 1

            # Start the vectors upon which residual sum of square and
row count data will be added
            rss <- c(0)
            seq <- c(0)

            # START THE ACTUAL two-part fit
            for (i in 1:loop)
                  {

                  fit1 <- lm(CO2~O2, data=Trimmedset[1:count+ignore,])
#fit model for 1st part of CO2:O2 curve
                  res1 <- fit1$residuals
                  resdf1 <- as.data.frame(res1)
                  rss1<-sum((resdf1$res1-mean(resdf1$res1))^2)
#calculate residual sum of squares for 1st part
```

```
                fit2 <- lm(CO2~O2,
data=Trimmedset[count+ignore+1:length(Dataset$CO2),]) #fit model for
2nd part of CO2:O2 curve
                res2 <- fit2$residuals
                resdf2 <- as.data.frame(res2)
                rss2<-sum((resdf2$res2-mean(resdf2$res2))^2)
#calculate residual sum of squares for 2nd part

                rss <- c(rss, rss1+rss2)
                seq <- c(seq, count) # arrange both RSS from this run
and the number from this run into vectors

                count <- count+1 # start the next run one data point
after

                }

          # Remove the initial zeroes used to start the vectors
          rss <- rss[2:length(rss)]
          seq <- seq[2:length(seq)]

          # Put the two vectors into a data frame
          compare <- data.frame(rss,seq)

          # Mark the seq value at minimum rss, add the ignore term to
match it with the original dataset's row number. O2 at the separation
is recorded for comparison with O2 at intersection, to check whether
the graph meets script assumptions.
          separate <-
((subset(compare,compare$rss==min(compare$rss)))$seq) + ignore
          separateO2 <- Trimmedset$O2[separate]

          # Fit model for the two regression lines determined to best
represent the division between the two parts
          finalfit1 <- lm(CO2~O2, data=Trimmedset[1:separate,])
          finalfit2 <- lm(CO2~O2,
data=Trimmedset[separate+1:length(Trimmedset$CO2),])

          # Pull out important values for easy commands
          slope1<-finalfit1$coefficients[[2]]
          inter1<-finalfit1$coefficients[[1]]
          slope2<-finalfit2$coefficients[[2]]
          inter2<-finalfit2$coefficients[[1]]

          # Calculate O2 and CO2 at intersection of the two lines
          finalo2<-(inter2-inter1)/(slope1-slope2)
          finalco2<-finalo2*slope1+inter1
```

```
            # Retrieve important values and combine into the
established vectors
            interrow <- c(interrow, separate)
            sepO2 <- c (sepO2, separateO2)
            interO2 <- c(interO2, finalo2)
            interCO2 <- c(interCO2, finalco2)
            line1slope <- c(line1slope, slope1)
            line1intercept <- c(line1intercept, inter1)
            line1r2 <- c(line1r2, summary(finalfit1)$r.squared)
            line2slope <- c(line2slope, slope2)
            line2intercept <- c(line2intercept, inter2)
            line2r2 <- c(line2r2, summary(finalfit2)$r.squared)

            # Plot and save graph
            plot(Trimmedset$O2[1:separate],Trimmedset$CO2[1:separate],
xlim = range(Trimmedset$O2), ylim = range (Trimmedset$CO2), col="red4",
xlab="Oxygen level (kPa)", ylab="Carbon dioxide level (ppm)")
            points(Trimmedset$O2[separate+1:length(Trimmedset$O2)],
Trimmedset$CO2[separate+1:length(Trimmedset$CO2)], col="blue4")
            abline(finalfit1, lwd = 2, col="red")
            abline(finalfit2, lwd = 2, col="blue")
            savePlot(Filenames$graphpaths[filecount], type = "pdf")
            savePlot(Filenames$pngpaths[filecount], type = "png")

            # Switch to next file for next loop if it's not the last
file yet
            if (filecount < length(Filenames$NAME))
                  {
                        filecount <- filecount+1
                  }

      }

    # Remove the initial zeroes used to start the key result vectors
    interrow <- interrow[2:length(interrow)]
    sepO2 <- sepO2[2:length(sepO2)]
    interO2 <- interO2[2:length(interO2)]
    interCO2 <- interCO2[2:length(interCO2)]
    line1slope <- line1slope[2:length(line1slope)]
    line2slope <- line2slope[2:length(line2slope)]
    line1intercept <- line1intercept[2:length(line1intercept)]
    line2intercept <- line2intercept[2:length(line2intercept)]
    line1r2 <- line1r2[2:length(line1r2)]
    line2r2 <- line2r2[2:length(line2r2)]
```

```
    # Create the final result table, saved as finalresults.csv in the
source folder
    Finalset <- data.frame(Filenames$NAME, interrow, sepO2, interO2,
interCO2, line1slope, line1intercept, line1r2, line2slope,
line2intercept, line2r2, Filenames$sourcepaths, Filenames$savepaths)
    colnames(Finalset) <- c("file name", "last row in line 1", "O2 at
separation", "O2 at intersection", "CO2 at intersection", "line 1
slope", "line 1 intercept", "line 1 R2", "line 2 slope", "line 2
intercept", "line 2 R2", "source file path", "trimmed save path")
    finalsavepath <- paste(path, "finalresults.csv", sep = "")
    write.table(Finalset, finalsavepath, sep=",", col.names=TRUE,
row.names=FALSE, quote=TRUE, na="NA")
```

Appendix II-A: Template for the data files used for the script in Appendix I.

The file was an Acqknowledge 3.8.1 data file later saved as a .txt file by the program. The .txt file saves the data as tab-separated values (TSV), having data channels as columns separated by the tab character (Tab key on keyboard) without a column header. In this case, the columns represent: oxygen analyzer reading (%), CO_2 analyzer reading (ppm), temperature reading (°C), activity signal (volt), and the root mean square (RMS) of recent activity, calculated with the past 40 data points and used as a visual cue for activity during trials.

20.99	3.23	25.17	0.003052	0.00241
20.97	3.35	25.16	0.002136	0.002328
20.97	2.61	25.13	0.002136	0.002265
20.97	3.29	25.15	0.001526	0.002206
20.96	3.10	25.17	0.003052	0.002168
20.98	3.53	25.15	0.000305	0.002111
20.96	3.23	25.18	0.005188	0.002132
20.98	2.79	25.17	0.003357	0.002145
20.97	2.12	25.15	0.00824	0.002098
20.97	2.12	25.13	0.001831	0.002325
20.99	3.41	25.12	0.000916	0.002291

Appendix II-B: Template for the list of data files (files.csv) used in the script.

The file included one column with the header “NAME” in the first row, followed by file names for the data files, without the .txt extension.

NAME
larvaPcrit25-20100820
larvaPcrit25-20100831
larvaPcrit25-20100910
larvaPcrit25-20100915
larvaPcrit25-20100924

Appendix III: Complete AIC tables for regression model selection.

Regression was between gall oxygen levels measured in the field (Chapter 4) and potential factors that influence gall oxygen level. First and last steps were shown in Table 4-2, and all steps are shown here. Calculated in R 2.12.0.

Parameter removed	K[a]	RSS	AIC_C[b]	Δ_i[c]	w_i[d]
dryratio	11	3085.2	465.17	**0**	0.140
amass	11	3085.9	465.20	0.03	0.138
temperature	11	3090.0	465.39	0.22	0.125
date	11	3091.1	465.44	0.27	0.122
ppt	11	3091.7	465.47	0.30	0.120
afly	11	3101.8	465.93	0.76	0.096
diam	11	3114.2	466.51	1.34	0.071
phigh	11	3126.9	467.09	1.92	0.053
ghigh	11	3133.3	467.38	2.21	0.046
None	12	3085.2	467.54	2.37	0.043
penetrate	11	3149.9	468.14	2.97	0.032
idiam	11	3186.5	469.79	4.62	0.014
year	11	3423.5	479.17	14.00	< 0.001

[a] Number of parameters

[b] AIC values with small sample size correction (AIC_C)

[c] Difference between the minimum AIC_C and the AIC_C achieved by removing the parameter in the current row. Δi of 0 indicates removing the current row's parameter leads to the minimum AIC_C.

[d] Akaike weight, interpreted as the probability that the current model is the best.

(Tables for subsequent steps continued for the following 3 pages)

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
amass	10	3202.9	478.65	**0**	0.152
temperature	10	3204.8	478.74	0.09	0.145
ppt	10	3206.1	478.80	0.15	0.141
afly	10	3207.6	478.87	0.22	0.136
date	10	3209.0	478.93	0.28	0.132
ghigh	10	3238.6	480.29	1.64	0.067
phigh	10	3240.1	480.36	1.71	0.065
diam	10	3252.9	480.94	2.29	0.048
None	11	3202.9	480.98	2.335	0.047
idiam	10	3260.5	481.29	2.64	0.041
penetrate	10	3279.1	482.13	3.48	0.027
year	10	3659.6	499.43	20.78	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
temperature	9	3204.8	476.43	**0**	0.173
ppt	9	3206.1	476.49	0.06	0.168
date	9	3209.0	476.62	0.19	0.157
afly	9	3208.9	476.62	0.19	0.157
ghigh	9	3238.6	477.98	1.55	0.080
phigh	9	3240.1	478.05	1.62	0.077
diam	9	3253.1	478.64	2.21	0.057
None	10	3202.9	478.65	2.211	0.057
idiam	9	3267.4	479.29	2.86	0.041
penetrate	9	3279.2	479.82	3.39	0.032
year	9	3685.7	497.12	20.69	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
ppt	8	3208.4	474.33	**0**	0.198
date	8	3209.1	474.37	0.04	0.194
afly	8	3209.9	474.40	0.07	0.191
ghigh	8	3239.2	475.75	1.42	0.097
phigh	8	3240.2	475.79	1.46	0.095
None	9	3204.8	476.43	2.108	0.069
diam	8	3254.4	476.44	2.11	0.069
idiam	8	3268.9	477.10	2.77	0.049
penetrate	8	3280.3	477.62	3.29	0.038
year	8	3712.1	495.92	21.59	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
afly	7	3213.2	472.32	**0**	0.243
date	7	3215.2	472.41	0.09	0.232
ghigh	7	3244.5	473.75	1.43	0.119
phigh	7	3246.8	473.86	1.54	0.113
None	8	3208.4	474.33	2.006	0.089
diam	7	3257.2	474.33	2.01	0.089
idiam	7	3271.8	474.99	2.67	0.064
penetrate	7	3282.3	475.46	3.14	0.051
year	7	3712.6	493.70	21.38	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
date	6	3217.3	470.30	**0**	0.326
ghigh	6	3252.5	471.92	1.62	0.145
phigh	6	3255.4	472.04	1.74	0.137
None	7	3213.2	472.32	2.024	0.119
diam	6	3263.0	472.39	2.09	0.115
idiam	6	3271.9	472.79	2.49	0.094
penetrate	6	3288.3	473.53	3.23	0.065
year	6	3723.4	491.93	21.63	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
ghigh	5	3252.6	469.74	**0**	0.229
phigh	5	3255.4	469.87	0.13	0.215
None	6	3217.3	470.30	0.553	0.174
idiam	5	3272.2	470.63	0.89	0.147
diam	5	3275.8	470.79	1.05	0.136
penetrate	5	3289.8	471.42	1.68	0.099
year	5	3724.1	489.78	20.04	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
phigh	4	3256.3	467.77	**0**	0.532
None	5	3252.6	469.74	1.973	0.198
idiam	4	3330.9	471.12	3.35	0.100
diam	4	3335.9	471.34	3.57	0.089
penetrate	4	3340.5	471.55	3.78	0.080
year	4	4214.7	505.95	38.18	< 0.001

Parameter removed	K	RSS	AIC_C	Δ_i	w_i
None	4	3256.3	467.77	**0**	0.473
idiam	3	3343.0	469.55	1.777	0.194
diam	3	3347.1	469.73	1.957	0.178
penetrate	3	3353.3	470.00	2.227	0.155
year	3	4266.5	505.65	37.877	< 0.001